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**(71) Applicant** (for all designated States except US): **COLEY PHARMACEUTICAL GMBH** [DE/DE]; Elisabeth-Selbert-Strasse 9, 40764 Langenfeld (DE).

**(72) Inventors; and**

**(75) Inventors/Applicants** (for US only): **BAUER, Stefan** [DE/DE]; Waisenhausstrasse 13, 80637 Munchen (DE). **LIPFORD, Grayson** [US/US]; Innere Wiener Strasse 7A, 81667 Munchen (DE). **WAGNER, Hermann** [DE/DE]; Kaagangerstrasse 36, 82279 Eching (DE).

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**(54) Title:** PROCESS FOR HIGH THROUGHPUT SCREENING OF CpG-BASED IMMUNO-AGONIST/ANTAGONIST

**(57) Abstract:** The invention pertains to murine TLR9 and related TLR9s which include murine-specific amino acids, as well as nucleic acids which encode those polypeptides. The present invention also includes fragments and biologically functional variants of the murine TLR9. The invention further relates to methods of using such murine and non-murine TLR9 nucleic acids and polypeptides, especially in methods for screening for agonists and antagonists of immunostimulatory CpG nucleic acids. Also included are murine TLR9 inhibitors which inhibit murine TLR9 activity by inhibiting the expression or function of murine TLR9. In a further aspect the present invention pertains to murine TLR7 and murine TLR8, as well as related TLR7 and TLR8 molecules which include murine-specific amino acids, as well as nucleic acids which encode those polypeptides. The present invention also includes fragments and biologically functional variants of the murine TLR7 and TLR8. Methods are included for screening for ligands of TLR7 and TLR8, as well as for inhibitors and agonists and antagonists of signaling mediated by TLR7 and TLR8.

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**PROCESS FOR HIGH THROUGHPUT SCREENING OF CpG-BASED  
IMMUNO-AGONIST/ANTAGONIST**

**Related Applications**

5 This invention claims benefit of U.S. Provisional Application 60/233,035, filed September 15, 2000; U.S. Provisional Application 60/263,657, filed January 23, 2001; U.S. Provisional Application 60/291,726, filed May 17, 2001; and U.S. Provisional Application 60/300,210, filed June 22, 2001.

10 **Field of the Invention**

The invention pertains to signal transduction by immunostimulatory nucleic acids.

**Background of the Invention**

Bacterial DNA is a potent immunomodulatory substance. Yamamoto S et al.,  
15 *Microbiol Immunol* 36:983-997 (1992). It has been hypothesized to be a pathogen-derived ligand recognized by an unidentified pathogen recognition receptor that initiates a host of innate and adaptive immune responses. Wagner H, *Adv Immunol* 73:329-368 (1999). CpG motif-containing oligodeoxynucleotides (CpG ODN) can mimic the biology of bacterial DNA. Krieg AM et al., *Nature* 374:546-549 (1995). CpG ODN and DNA vectors have  
20 recently been shown to be of clinical value due to immunostimulatory, hematopoietic and adjuvant qualities.

The adaptive immune system appeared approximately 450 million years ago when a transposon that carried the forerunners of the recombinase activating genes, RAG-1 and RAG-2, was inserted into the germ line of early jawed vertebrates. Agarwal A. et al., *Nature* 25 394:744 (1998). The ability to mount an adaptive immune response allowed organisms to remember the pathogens that they had already encountered, and natural selection made the adaptive immune response a virtually universal characteristic of vertebrates. However, this did not lead to discarding the previous form of host defense, the innate immune system. Indeed, this earlier form of host defense has been coopted to serve a second function, stimulating and orienting the primary adaptive immune response by controlling the expression of costimulatory molecules.  
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It had been surmised for a decade that cells of the innate immune system bear receptors for conserved molecular patterns associated with microbial pathogens. According to this model, when the protein antigens derived from pathogens are processed and presented as peptides that serve as the stimulus for specific T cell receptors, pattern recognition

5 receptors (PRRs) on the antigen-presenting cells also induce the synthesis of costimulatory molecules, cytokines, and chemokines. These activated antigen-presenting cells serve to attract and activate the antigen-specific T cells that are essential to all adaptive immune responses. Janeway CAJ, *Cold Spring Harbor Symp Quant Biol* 54:1 (1989); Fearon DT et al., *Science* 272:50 (1996); and Medzhitov R et al., *Cell* 91:295 (1997). It was known that the

10 substances that can induce costimulation include bacterial lipopolysaccharide (LPS), synthetic double-stranded RNA, glycans, and mannans. Furthermore, experimental evidence indicated that the processed antigen ligand for the T cell had to be on the same cell as the costimulatory molecule. This is obviously of crucial importance for maintaining self-tolerance; bystander presentation of costimulatory molecules would mean that tolerance would be lost whenever

15 an infection occurred.

To validate this model, it was necessary to identify receptors for microbial patterns that, upon binding pathogen ligands, initiate signaling cascades leading to the production of costimulatory molecules and cytokines. Molecules such as mannose binding protein (MBP) do not qualify for this role, because they activate proteolytic cascades or promote

20 phagocytosis but are not known to induce costimulation. The break-through came with the identification of a human homologue of *Drosophila* Toll initially cloned as a cDNA and later named hTLR4 (for human Toll-like receptor). Medzhitov R et al., *Nature* 388:394 (1997); Rock FL et al., *Proc Natl Acad Sci USA* 95:588 (1998); Chaudhary PM et al., *Blood* 91:4020-4027 (1998).

25 Toll-like receptors (TLRs) are a family of germline-encoded transmembrane proteins that facilitate pathogen recognition and activation of the innate immune system. Hoffmann JA et al., *Science* 284, 1313-1318 (1999); Rock FL et al., *Proc Natl Acad Sci USA* 95:588-593 (1998). TLRs engage conserved pathogen-derived ligands and subsequently activate the TLR/IL-1R signal transduction pathway to induce a variety of effector genes. Medzhitov R et al., *Mol Cell* 2:253-258 (1998); Muzio M et al., *J Exp Med* 187:2097-2101 (1998).

So far, ten different mammalian TLRs have been described. Rock FL et al., *Proc Natl*

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Acad Sci USA 95:588-593 (1998); Chaudhary PM et al., *Blood* 91:4020-4027 (1998); Takeuchi O et al., *Gene* 231:59-65 (1999); Aderem A. et al., *Nature* 406:782-7 (2000). So far, genetic data suggest that the TLRs have unique functions and are not redundant. Ligands for and the function of most of these TLRs, aside from TLR2 and TLR4, remain to be elucidated.

It turns out that an LPS-binding and signaling receptor complex is assembled when hTLR4 interacts with LPS bound to CD14, a peripheral membrane protein held to the cell surface by a glycosyl-phosphoinositol tail. The presence of LPS binding protein (LBP) further increases signaling. The hTLR4 protein has a leucine-rich repeat sequence in its extracellular domain that interacts with CD14 complexed with LPS. TLR4 then transduces the LPS signal across the membrane because destructive mutation of this gene lead to an LPS-unresponsive state in mice, which are also deficient in the clearance of Gram-negative bacteria. Poltorak A et al., *Science* 282:2085 (1998); Qureshi ST et al., *J Exp Med* 189:615-625 (1999); Eden CS et al., *J Immunol* 140:180 (1988). It has since become apparent that humans, like flies, have numerous Toll-like receptors (TLRs).

TLR4 and other TLRs have a cytoplasmic Toll/IL-1 receptor (TIR) homology domain. This domain communicates with a similar domain on an adapter protein (MyD88) that interacts with TLR4 by means of a like:like interaction of TIR domains. The next interaction is between the adapter and a kinase, through their respective "death domains." The kinase in turn interacts with tumor necrosis factor (TNF) receptor-associated factor-6 (TRAF6). Medzhitov R et al., *Mol Cell* 2:253 (1998); Kopp EB et al., *Curr Opin Immunol* 11:15 (1999). After TRAF6, two sequential kinase activation steps lead to phosphorylation of the inhibitory protein I $\kappa$ B and its dissociation from NF- $\kappa$ B. The first kinase is a mitogen-activated kinase kinase kinase (MAPKKK) known as NIK, for NF- $\kappa$ B-inducing kinase. The target of this kinase is another kinase made up of two chains, called I $\kappa$ B kinase  $\alpha$  (IKK $\alpha$ ) and I $\kappa$ B kinase  $\beta$  (IKK $\beta$ ), that together form a heterodimer of IKK $\alpha$ :IKK $\beta$ , which phosphorylates I $\kappa$ B. NF- $\kappa$ B translocates to the nucleus to activate genes with  $\kappa$ B binding sites in their promoters and enhancers such as the genes encoding interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-6, IL-8, the p40 protein of IL-12, and the costimulatory molecules CD80 and CD86.

The types of cells that respond to CpG DNA - B cells, dendritic cells (DCs) and macrophages - are also stimulated by other pathogen-derived pattern-recognition factors, such

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as LPS. In general, the PRRs of the innate immune system are situated on the cell surface, where they are probably best able to detect microbes. Although cell-surface proteins that bind

DNA are well described, and have been proposed to mediate immune activation by CpG motif (Liang H et al., *J Clin Invest* 98:1119-1129 (1998)), this binding is sequence-

5 independent and does not bring about cell activation. Krieg AM et al., *Nature* 374:546-549 (1995); Yamamoto T et al., *Microbiol Immunol* 38:831-836 (1994); Häcker H et al., *EMBO J* 17:6230-6240 (1998). Because CpG ODNs that have been immobilized to prevent cell uptake are nonstimulatory (Krieg AM et al., *Nature* 374:546-549 (1995); Manzel L et al., *Antisense Nucleic Acid Drug Dev* 9:459-464 (1999)), it appears that CpG ODN probably

10 work by binding to an intracellular receptor. In support of this hypothesis, drugs such as chloroquine, which interfere with the endosomal acidification/processing of ODNs, specifically block the immune stimulatory effects of CpG DNA. Häcker H et al., *EMBO J* 17:6230-6240 (1998); Macfarlane DE et al., *J Immunol* 160:1122-1131 (1998); Yi AK et al., *J Immunol* 160:4755-4761 (1998). It has been proposed that an endosomal step is required

15 for the CpG-induced signal transduction pathways. Häcker H et al., *EMBO J* 17:6230-6240 (1998); Yi AK et al., *J Immunol* 160:4755-4761 (1998). How the information contained in unmethylated CpG-motifs of bacterial DNA trigger changes in gene expression has not previously been discovered.

Since the receptor for bacterial DNA has been unknown, development of screening for

20 optimal CpG motifs through direct binding analysis has been limited. An additional complication appears to be species-specific selectivity for CpG sequence, i.e., an optimal sequence for one species may not be optimal for another.

### Summary of the Invention

25 Nucleic acids encoding three Toll-like receptors, Toll-like receptor 7 (TLR7), TLR8, and TLR9 of the mouse have now been identified, isolated, cloned and sequenced by the inventors. The invention in general provides isolated nucleic acid molecules encoding TLRs and isolated fragments of those nucleic acid molecules; isolated TLR polypeptides and isolated fragments of those polypeptides; expression vectors containing the foregoing nucleic acid molecules; host cells having the foregoing expression vectors; fusion proteins including the TLR polypeptides and fragments thereof; and screening methods useful for identifying,

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comparing, and optimizing agents which interact with these TLRs, particularly agents that alter the expression of and signaling associated with these TLR molecules. In preferred embodiments the screening methods are high throughput screening methods.

The invention in some aspects arises from the surprising discovery that TLR9 is involved in immunostimulatory nucleic acid (ISNA)-induced immunostimulation. The invention also stems in part from the surprising discovery that TLR9 transduces immune activating signals in response to ISNA in a manner that is both sequence-specific and species-specific.

In a first aspect the invention provides isolated nucleic acid molecules which encode full-length murine TLR9. According to this aspect of the invention, isolated nucleic acid molecules are provided which are selected from the group consisting of (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence set forth as SEQ ID NO:1, and which code for a murine TLR9 having an amino acid sequence set forth as SEQ ID NO:3; (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code; and (c) complements of (a) or (b). In a certain embodiment, the isolated nucleic acid molecule codes for SEQ ID NO:3, where SEQ ID NO:3 represents the deduced amino acid sequence of full-length murine TLR9. In some embodiments the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:1 or SEQ ID NO:2, where these correspond to full-length cDNA and the open reading frame for murine TLR9, respectively.

The term "stringent conditions" as used herein refers to combined conditions based on parameters including salt, temperature, organic solvents, and optionally other factors with which the practitioner skilled in the art is familiar. Nucleic acid hybridization parameters may be found in references which compile such methods, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. More specifically, stringent conditions, as used herein, refers, for example, to hybridization at 65°C in hybridization buffer (3.5 x SSC, 0.02% Ficoll, 0.02% polyvinyl pyrrolidone, 0.02% bovine serum albumin, 2.5mM NaH<sub>2</sub>PO<sub>4</sub> (pH7), 0.5% SDS, 2mM EDTA). SSC is 0.15M sodium chloride/0.15M sodium citrate, pH7; SDS is sodium dodecyl sulfate; and EDTA is ethylenediaminetetraacetic

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acid. After hybridization, the membrane upon which the DNA is transferred is washed with 2 x SSC at room temperature and then with 0.1 - 0.5 x SSC/0.1 x SDS at temperatures up to 68°C. There are other conditions, reagents, and so forth which can be used, which result in a similar degree of stringency. The skilled artisan will be familiar with such conditions, and thus they are not given here. It will be understood, however, that the skilled artisan will be able to manipulate the conditions in a manner to permit the clear identification of alleles of murine TLR nucleic acids of the invention. The skilled artisan also is familiar with the methodology for screening cells and libraries for expression of such molecules which then are routinely isolated, followed by isolation of the pertinent nucleic acid molecule and sequencing.

The invention in a second aspect provides isolated TLR9 polypeptides or fragments thereof. The isolated TLR9 polypeptides or fragments thereof include at least one amino acid of a murine TLR9 selected from the group consisting of amino acids 2, 3, 4, 6, 7, 18, 19, 22, 38, 44, 55, 58, 61, 62, 63, 65, 67, 71, 80, 84, 87, 88, 91, 101, 106, 109, 117, 122, 123, 134, 136, 140, 143, 146, 147, 157, 160, 161, 167, 168, 171, 185, 186, 188, 189, 191, 199, 213, 217, 220, 227, 231, 236, 245, 266, 269, 270, 271, 272, 273, 274, 278, 281, 285, 297, 298, 301, 305, 308, 311, 322, 323, 325, 326, 328, 332, 335, 346, 348, 353, 355, 358, 361, 362, 365, 367, 370, 372, 380, 381, 382, 386, 389, 392, 394, 397, 409, 412, 413, 415, 416, 419, 430, 432, 434, 435, 438, 439, 443, 444, 446, 447, 448, 450, 451, 452, 454, 455, 459, 460, 463, 465, 466, 468, 469, 470, 472, 473, 474, 475, 478, 488, 489, 494, 495, 498, 503, 508, 510, 523, 531, 539, 540, 543, 547, 549, 561, 563, 565, 576, 577, 579, 580, 587, 590, 591, 594, 595, 597, 599, 601, 603, 610, 611, 613, 616, 619, 632, 633, 640, 643, 645, 648, 650, 657, 658, 660, 667, 670, 672, 675, 679, 689, 697, 700, 703, 705, 706, 711, 715, 716, 718, 720, 723, 724, 726, 729, 731, 735, 737, 743, 749, 750, 751, 752, 754, 755, 759, 760, 772, 774, 780, 781, 786, 787, 788, 800, 814, 821, 829, 831, 832, 835, 844, 857, 858, 859, 862, 864, 865, 866, 879, 893, 894, 898, 902, 910, 917, and 927 of SEQ ID NO:3, wherein the TLR9 polypeptide or fragment thereof has an amino acid sequence which is identical to a human TLR9 polypeptide or fragment thereof except for the at least one amino acid of murine TLR9. The TLR9 polypeptide or fragment thereof in certain embodiments according to this aspect of the invention further includes at least one amino acid of murine TLR9 selected from the group consisting of amino acids 949, 972, 975, 976, 994, 997, 1000, 1003, 1004, 1010,

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1011, 1018, 1023, and 1027 of SEQ ID NO:3. Thus specifically excluded from this aspect of the invention are TLR9 fragments restricted to the C-terminal 95 amino acids and fragments thereof.

In certain embodiments the TLR9 polypeptide and fragments thereof according to this aspect of the invention exclude those TLR9 polypeptides and fragments thereof which differ from human TLR9 and fragments thereof only by one or more conservative amino acid substitutions at particular sites noted above. As is well known in the art, a "conservative amino acid substitution" refers to an amino acid substitution which generally does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Conservative substitutions of amino acids typically include substitutions made amongst amino acids within the following groups: methionine (M), isoleucine (I), leucine (L), valine (V); phenylalanine (F), tyrosine (Y), tryptophan (W); lysine (K), arginine (R), histidine (H); alanine (A), glycine (G); serine (S), threonine (T); glutamine (Q), asparagine (N); and glutamic acid (E), aspartic acid (D).

According to this and other aspects of the invention, with reference to TLR "polypeptides and fragments thereof," "fragments thereof" refers to polypeptide fragments having stretches of contiguous amino acid residues that are at least about 8 amino acids long. Generally the fragments are at least about 10 amino acids long; more generally at least 12 amino acids long; often at least about 14 amino acids long; more often at least about 16 amino acids long; typically at least 18 amino acids long; more typically at least 20 amino acids long; usually at least 22 amino acids long; and more usually at least 24 amino acids long. Certain preferred embodiments include larger fragments that are, for example, at least about 30 amino acids long, at least about 40 amino acids long, at least about 50 amino acids long, at least about 100 amino acids long, at least about 200 amino acids long, and so on, up to and including fragments that are a single amino acid shorter than full-length TLR polypeptide.

In certain embodiments, the human TLR9 has an amino acid sequence set forth as SEQ ID NO:6.

In preferred embodiments, the isolated TLR9 polypeptides or fragments thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:3 and fragments of SEQ ID NO:3. In some embodiments according to this aspect of the invention,

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the isolated TLR9 polypeptides or fragments thereof include combinations of the foregoing human and murine TLR9 polypeptides.

In certain preferred embodiments the isolated TLR9 polypeptide or fragment thereof is an extracytoplasmic domain (also referred to herein as extracellular domain) of TLR9, or a portion thereof. As described in greater detail further herein, TLR7, TLR8, and TLR9 have certain structural and functional domains. Structural domains of these TLRs include but are not limited to an extracytoplasmic domain, a transmembrane domain, and a cytoplasmic domain. The extracytoplasmic domain extends into the lumen of endosomal/lysosomal vesicles. The cytoplasmic domain includes a Toll/interleukin-1 receptor-like domain (also referred to as Toll/IL-1R domain, TIR homology domain, or TIR domain). In murine TLR9 the extracytoplasmic, transmembrane, and cytoplasmic domains correspond to amino acids 1 to about 819, about 820 to about 837, and about 838 to about 1032, respectively.

As mentioned above, it has been discovered according to the invention that TLR9 is involved in immune activation induced by certain nucleic acid molecules referred to in the art as immunostimulatory nucleic acids (ISNAs), including CpG nucleic acids. It is believed by the inventors that binding of ISNA to TLR9 leads to signal transduction involving the TIR domain of TLR9. Thus in certain embodiments according to this aspect of the invention, the isolated TLR9 polypeptide or fragment thereof selectively binds to an ISNA, including an ISNA that is a CpG nucleic acid.

Also included according to this aspect of the invention are isolated TLR9 polypeptides or fragments thereof which are portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids such as CpG nucleic acids. In certain embodiments such portions include an MBD motif set forth as any one of SEQ ID NOS: 126, 127, 210, and 211. In certain embodiments portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids include a CXXC motif set forth as any one of SEQ ID NOS: 196, 197, and 198.

According to a third aspect of the invention, isolated nucleic acid molecules are provided which encode the foregoing isolated TLR9 polypeptides or fragments thereof. The isolated nucleic acid molecules according to this aspect of the invention specifically exclude certain expressed sequence tags (ESTs) identified by the following GenBank accession numbers: AA162495, AA197442, AA273731, AA794083, AA915125, AA968074,

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AI428529, AI451215, AI463056, AI893951, AV142833, AV326033, AV353853,  
AW048117, AW048548, AW215685, AW549817, BB179985, BB215203, BB283380,  
BB285606, BB312895, BB497196, BB622397, BF016670, BF150116, BF161011,  
BF300296, BF385702, BF539367, BF784415, BG863184, BG922959, BG967012,  
5 BG974917, BI105291, BI153921, BI651868, BI653892, and W76964.

In a fourth aspect the invention provides isolated nucleic acid molecules which encode full-length murine TLR7. According to this aspect of the invention, isolated nucleic acid molecules are provided which are selected from the group consisting of (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a 10 nucleotide sequence set forth as SEQ ID NO:173, and which code for a murine TLR7 having an amino acid sequence set forth as SEQ ID NO:175; (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code; and (c) complements of (a) or (b). In a certain embodiment, the isolated nucleic acid molecule codes for SEQ ID NO:175, where SEQ ID NO:175 represents the deduced amino 15 acid sequence of full-length murine TLR7. In some embodiments the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:173 or SEQ ID NO:174, where these correspond to full-length cDNA and the open reading frame for murine TLR7, respectively.

The invention in a fifth aspect provides isolated TLR7 polypeptides or fragments 20 thereof. The isolated TLR7 polypeptides or fragments thereof include at least one amino acid of a murine TLR7 selected from the group consisting of amino acids 4, 8, 15, 16, 18, 21, 23, 24, 25, 27, 37, 39, 40, 41, 42, 44, 45, 61, 79, 83, 86, 89, 92, 96, 103, 109, 111, 113, 119, 121, 127, 128, 131, 145, 148, 151, 164, 172, 176, 190, 202, 203, 204, 205, 222, 225, 226, 228, 236, 238, 243, 250, 253, 266, 268, 271, 274, 282, 283, 287, 288, 308, 313, 314, 315, 325, 25 328, 331, 332, 341, 343, 344, 347, 351, 357, 360, 361, 362, 363, 364, 365, 366, 370, 371, 377, 378, 387, 388, 389, 392, 397, 398, 413, 415, 416, 419, 421, 422, 425, 437, 438, 440, 446, 449, 453, 454, 455, 456, 462, 470, 482, 486, 487, 488, 490, 491, 493, 494, 503, 505, 509, 511, 529, 531, 539, 540, 543, 559, 567, 568, 574, 583, 595, 597, 598, 600, 611, 613, 620, 624, 638, 645, 646, 651, 652, 655, 660, 664, 665, 668, 669, 672, 692, 694, 695, 698, 30 701, 704, 714, 720, 724, 727, 728, 733, 738, 745, 748, 755, 762, 777, 780, 789, 803, 846, 850, 851, 860, 864, 868, 873, 875, 884, 886, 888, 889, 890, 902, 903, 911, 960, 967, 970,

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980, 996, 1010, 1018, 1035, and 1045 of SEQ ID NO:175, wherein the TLR7 polypeptide or fragment thereof has an amino acid sequence which is identical to a human TLR7 polypeptide or fragment thereof except for the at least one amino acid of murine TLR7.

In certain embodiments the TLR7 polypeptide and fragments thereof according to this 5 aspect of the invention exclude those TLR7 polypeptides and fragments thereof which differ from human TLR7 and fragments thereof only by one or more conservative amino acid substitutions at particular sites noted above.

In certain embodiments, the human TLR7 has an amino acid sequence set forth as SEQ ID NO:170.

10 In preferred embodiments, the isolated TLR7 polypeptides or fragments thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:175 and fragments of SEQ ID NO:175. In some embodiments according to this aspect of the invention, the isolated TLR7 polypeptides or fragments thereof include combinations of the foregoing human and murine TLR7 polypeptides.

15 In certain preferred embodiments the isolated TLR7 polypeptide or fragment thereof is an extracytoplasmic domain of TLR7, or a portion thereof. In certain embodiments according to this aspect of the invention, the isolated TLR7 polypeptide or fragment thereof selectively binds to an ISNA, including an ISNA that is a CpG nucleic acid. Also included according to this aspect of the invention are isolated TLR7 polypeptides or fragments thereof which are 20 portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids such as CpG nucleic acids. In certain embodiments such portions include an MBD motif set forth as any one of SEQ ID NOs: 203, 204, 212, and 213. In certain embodiments portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids include a CXXC motif set forth as any one of 25 SEQ ID NOs: 196, 199, and 200.

According to a sixth aspect of the invention, isolated nucleic acid molecules are provided which encode the foregoing isolated TLR7 polypeptides or fragments thereof. The isolated nucleic acid molecules according to this aspect of the invention specifically exclude certain ESTs identified by the following GenBank accession numbers: AA176010, 30 AA210352, AA241310, AA266000, AA266744, AA276879, AA288480, AA871870, AI119722, AI449297, AI466859, AI604175, AV322307, BB033376, BB116163, BB210788,

- 11 -

BB464985, BB466708, BB636153, BF101884, BF124798, BF143871, BG067922, BG080980, BG082140, BG871070, BG964747, BG976560, BI150306, BI411471, and C87987.

In a seventh aspect the invention provides isolated nucleic acid molecules which encode full-length murine TLR8. According to this aspect of the invention, isolated nucleic acid molecules are provided which are selected from the group consisting of (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence set forth as SEQ ID NO:190, and which code for a murine TLR8 having an amino acid sequence set forth as SEQ ID NO:192; (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code; and (c) complements of (a) or (b). In a certain embodiment, the isolated nucleic acid molecule codes for SEQ ID NO:192, where SEQ ID NO:192 represents the deduced amino acid sequence of full-length murine TLR8. In some embodiments the isolated nucleic acid molecule comprises the nucleotide sequence of SEQ ID NO:190 or SEQ ID NO:191, where these correspond to full-length cDNA and the open reading frame for murine TLR8, respectively.

The invention in an eighth aspect provides isolated TLR8 polypeptides or fragments thereof. The isolated TLR8 polypeptides or fragments thereof include at least one amino acid of a murine TLR8 selected from the group consisting of amino acids 5, 6, 9, 10, 14, 15, 18, 21, 22, 23, 24, 25, 26, 27, 28, 30, 39, 40, 41, 43, 44, 50, 51, 53, 55, 61, 67, 68, 74, 80, 85, 93, 98, 99, 100, 104, 105, 106, 107, 110, 114, 117, 119, 121, 124, 125, 134, 135, 138, 145, 155, 156, 157, 160, 161, 162, 163, 164, 166, 169, 170, 174, 180, 182, 183, 186, 187, 191, 193, 194, 196, 197, 199, 200, 207, 209, 210, 227, 228, 230, 231, 233, 234, 241, 256, 263, 266, 267, 268, 269, 272, 274, 275, 276, 280, 285, 296, 298, 299, 300, 303, 305, 306, 307, 310, 312, 320, 330, 333, 335, 343, 344, 345, 346, 347, 349, 351, 356, 362, 365, 366, 375, 378, 379, 380, 381, 383, 384, 386, 387, 392, 402, 403, 408, 414, 416, 417, 422, 426, 427, 428, 429, 430, 431, 433, 437, 438, 439, 440, 441, 444, 445, 449, 456, 461, 463, 471, 483, 486, 489, 490, 494, 495, 496, 505, 507, 509, 512, 513, 519, 520, 523, 537, 538, 539, 541, 542, 543, 545, 554, 556, 560, 567, 569, 574, 575, 578, 586, 592, 593, 594, 595, 597, 599, 602, 613, 617, 618, 620, 621, 623, 628, 630, 633, 639, 641, 643, 644, 648, 655, 658, 661, 663, 664, 666, 668, 677, 680, 682, 687, 688, 690, 692, 695, 696, 697, 700, 702, 703, 706, 714,

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715, 726, 727, 728, 730, 736, 738, 739, 741, 746, 748, 751, 752, 754, 757, 764, 766, 772,  
776, 778, 781, 784, 785, 788, 791, 795, 796, 801, 802, 806, 809, 817, 820, 821, 825, 828,  
829, 831, 839, 852, 853, 855, 858, 863, 864, 900, 903, 911, 918, 934, 977, 997, 1003, 1008,  
1010, 1022, 1023, 1024, 1026, and 1030 of SEQ ID NO:192, wherein the TLR8 polypeptide  
5 or fragment thereof has an amino acid sequence which is identical to a human TLR8  
polypeptide or fragment thereof except for the at least one amino acid of murine TLR8.

In certain embodiments the TLR8 polypeptide and fragments thereof according to this aspect of the invention exclude those TLR8 polypeptides and fragments thereof which differ from human TLR8 and fragments thereof only by one or more conservative amino acid  
10 substitutions at particular sites noted above.

In certain embodiments, the human TLR8 has an amino acid sequence set forth as SEQ ID NO:184.

In preferred embodiments, the isolated TLR8 polypeptides or fragments thereof include an amino acid sequence selected from the group consisting of SEQ ID NO:192 and fragments of SEQ ID NO:192. In some embodiments according to this aspect of the invention, the isolated TLR8 polypeptides or fragments thereof include combinations of the foregoing human and murine TLR8 polypeptides.  
15

In certain preferred embodiments the isolated TLR8 polypeptide or fragment thereof is an extracytoplasmic domain of TLR8, or a portion thereof. In certain embodiments according to this aspect of the invention, the isolated TLR8 polypeptide or fragment thereof selectively binds to an ISNA, including an ISNA that is a CpG nucleic acid. Also included according to this aspect of the invention are isolated TLR8 polypeptides or fragments thereof which are portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids such as CpG nucleic acids. In certain embodiments such portions include an MBD motif set forth as any one of SEQ ID NOS: 205, 206, 214, and 215.  
20  
25 In certain embodiments portions of the extracytoplasmic domain believed by the inventors to interact with immunostimulatory nucleic acids include a CXXC motif set forth as any one of SEQ ID NOS: 196, 201, and 202.

According to a ninth aspect of the invention, isolated nucleic acid molecules are provided which encode the foregoing isolated TLR8 polypeptides or fragments thereof. The isolated nucleic acid molecules according to this aspect of the invention specifically exclude  
30

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certain ESTs identified by the following GenBank accession numbers: AA116795, AA268605, AA920337, AI529457, AI849892, AV097766, AV117427, AV164719, AV169968, AW551677, BB143750, BB214171, BB243478, BB244318, BB254686, BB256660, BB258368, BB278984, BB291470, BB292008, BB364655, BB373674, 5 BB428800, BB439876, BB444812, BB445724, BB465766, BB470182, BB535086, BB573907, BB573981, BB607650, BF135656, BF722808, BG299237, BG918020, BG919592, and W39977.

In a further aspect, the invention provides TLR expression vectors comprising the foregoing isolated nucleic acid molecules operably linked to a promoter. Thus in certain 10 embodiments pertaining to TLR9, the expression vector includes an isolated nucleic acid molecule according to the first aspect or the third aspect of the invention, operably linked to a promoter. In other embodiments, relating to TLR7, the expression vector includes an isolated nucleic acid molecule according to the fourth aspect or the sixth aspect of the invention, operably linked to a promoter. In yet other embodiments, relating to TLR8, the expression 15 vector includes an isolated nucleic acid molecule according to the seventh aspect or the ninth aspect of the invention, operably linked to a promoter.

The expression vectors according to this aspect of the invention are designed and constructed so that when they are introduced into a cell, under proper conditions they direct expression of the gene product encoded by the incorporated isolated nucleic acid molecule. 20 For example, the promoter can be constitutively active or it can be inducible or repressible upon interaction with a suitable inducer or repressor compound.

According to another aspect, host cells are provided that include a TLR expression vector of the invention. While any suitable method can be used, an expression vector typically is introduced into a cell by transfection or transformation. The host cells 25 transformed or transfected with the TLR expression vectors are in some embodiments co-transformed or co-transfected with another expression vector useful for the expression of another polypeptide. Alternatively, a host cell can be tranformed or transfected with an expression vector capable of directing expression of a TLR polypeptide or fragment thereof of the invention and (i) at least one additional TLR polypeptide or fragment thereof, or (ii) at 30 least one non-TLR polypeptide or fragment thereof. In certain preferred embodiments, the host cell includes separate expression vectors for any combination of TLR7, TLR8, and

TLR9. In some embodiments, a co-transformed or co-transfected expression vector may be useful for detection or regulation of TLR expression or TLR-related signaling. Specifically, in certain preferred embodiments the host cell includes an expression vector providing a reporter construct capable of interacting with a TIR domain.

5 In another aspect, the invention provides agents which selectively bind the isolated TLR polypeptides and fragments thereof of the invention. In certain embodiments the agent does not bind a human TLR polypeptide or fragment thereof, wherein the human TLR is selected from human TLR7, TLR8, and TLR9. In certain embodiments the agent is a polypeptide, preferably one selected from the group consisting of monoclonal antibodies, 10 polyclonal antibodies, Fab antibody fragments, F(ab')<sub>2</sub> antibody fragments, Fv antibody fragments, antibody fragments including a CDR3 region, and fusion proteins and other polypeptides including any such antibodies or antibody fragments.

Also provided are agents which selectively bind the foregoing isolated nucleic acid molecules, preferably antisense nucleic acid molecules which selectively bind to any of the 15 foregoing isolated nucleic acid molecules encoding a TLR polypeptide or fragment thereof. In some embodiments the agent is an isolated nucleic acid molecule which hybridizes under stringent conditions to an isolated nucleic acid molecule provided according to any of the first, third, fourth, fifth, sixth, and eighth aspects of the invention. In certain preferred embodiments the agent is an isolated nucleic acid molecule having a nucleotide sequence 20 which is complementary to an isolated nucleic acid molecule provided according to any of the first, third, fourth, fifth, sixth, and eighth aspects of the invention.

In still other aspects of the invention, methods for inhibiting TLR expression and TLR signaling in a cell are provided. The methods include contacting the cell with an amount of an agent effective to inhibit TLR expression and TLR signaling in the cell, wherein the TLR 25 is selected from the group consisting of TLR7, TLR8, and TLR9. In some embodiments the agent brought into contact with the cell is selected from the group consisting of monoclonal antibodies, polyclonal antibodies, Fab antibody fragments, F(ab')<sub>2</sub> antibody fragments, Fv antibody fragments, antibody fragments including a CDR3 region, and fusion proteins and other polypeptides that include any such antibodies or antibody fragments. In some 30 embodiments the cell is contacted with an antisense nucleic acid specific for the TLR, in an amount effective to inhibit TLR expression in the cell. In some embodiments the cell is

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contacted with an agent such as a cytokine or small molecule, in an amount effective to inhibit TLR expression in the cell.

In yet another aspect the invention provides a method for identifying nucleic acid molecules which interact with a TLR polypeptide or a fragment thereof. The method involves contacting a TLR polypeptide selected from the group consisting of TLR7, TLR8, TLR9, and nucleic acid-binding fragments thereof with a test nucleic acid molecule; and measuring an interaction of the test nucleic acid molecule with the TLR polypeptide or fragment thereof. Nucleic acid-binding fragments of TLRs preferably include the extracytoplasmic domain or subportions thereof, such as those which include at least an MBD motif, a CXXC motif, or both an MBD motif and a CXXC motif.

In this and other aspects of the invention involving methods of use of TLR polypeptides and fragments thereof, in some embodiments the TLR polypeptide or fragment thereof is TLR7. Likewise in this and other aspects of the invention involving methods of use of TLR polypeptides and fragments thereof, in some embodiments the TLR polypeptide or fragment thereof is TLR8. Also in this and other aspects of the invention involving methods of use of TLR polypeptides and fragments thereof, in some embodiments the TLR polypeptide or fragment thereof is TLR9.

In this and other aspects of the invention involving methods of use of TLR polypeptides and fragments thereof, in some embodiments the TLR polypeptide or fragment thereof is expressed in a cell. The cell expressing the TLR polypeptide or fragment thereof may naturally express the TLR polypeptide or fragment thereof, or it may be a host cell as provided by other aspects of the instant invention.

In this and other aspects of the invention involving methods of use of TLR polypeptides and fragments thereof, in some embodiments the TLR polypeptide or fragment thereof is an isolated TLR polypeptide or fragment thereof. In certain preferred embodiments the isolated TLR polypeptide or fragment thereof is immobilized on a solid support, for example a multiwell plate, a slide, a BIACore chip, a bead, a column, and the like. The immobilization can be accomplished by any chemical or physical method suitable for the purpose of the assay to be performed according to the method of the invention.

In certain embodiments the TLR polypeptide or fragment thereof is fused with an Fc fragment of an antibody. The Fc fragment portion of such a fusion molecule may be useful,

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for example, for attaching the TLR polypeptide or fragment thereof to a substrate, or for providing a target for detecting the presence of the TLR polypeptide or fragment thereof. The Fc fragment can be selected from any suitable vertebrate species and will typically, but not necessarily, be derived from an antibody belonging to the IgG class of antibodies. For  
5 example, the Fc can be a human or a murine Fcy. In certain embodiments the TLR polypeptide or fragment thereof is fused with an Fc fragment of an antibody with a specific cleavage site at or near the junction between the TLR polypeptide or fragment thereof and the Fc fragment. In one preferred embodiment the cleavage site is a thrombin protease recognition site. In a preferred embodiment the TLR polypeptide or fragment thereof fused  
10 with the Fc fragment includes a TLR extracytoplasmic domain.

In certain embodiments the interaction involving the TLR polypeptide or fragment thereof and the test nucleic acid molecule is binding between the TLR polypeptide or fragment thereof and the test nucleic acid molecule.

In certain embodiments according to this aspect of the invention, the measuring is  
15 accomplished by a method selected from the group consisting of enzyme-linked immunosorbent assay (ELISA), biomolecular interaction assay (BIA), electromobility shift assay (EMSA), radioimmunoassay (RIA), polyacrylamide gel electrophoresis (PAGE), and Western blotting.

In certain embodiments the measuring is accomplished by a method comprising  
20 measuring a response mediated by a TLR signal transduction pathway. For example, the response mediated by a TLR signal transduction pathway can be selected from the group consisting of induction of a gene under control of NF- $\kappa$ B promoter and secretion of a cytokine. In certain preferred embodiments the gene under control of NF- $\kappa$ B promoter is selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and  
25 TNF-luc. In certain preferred embodiments the secreted cytokine is selected from the group consisting of IL-8, TNF- $\alpha$ , and IL-12 p40.

In another embodiment the method according to this aspect of the invention can be used to determine if the test nucleic acid molecule is an immunostimulatory nucleic acid. The method involves the additional steps of comparing (a) the response mediated by a TLR signal transduction pathway as measured in the presence of the test nucleic acid molecule with (b) a response mediated by a TLR signal transduction pathway as measured in the absence of the

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test nucleic acid molecule; and determining the test nucleic acid molecule is an immunostimulatory nucleic acid when (a) exceeds (b).

In yet another embodiment the method according to this aspect of the invention can be used to determine if the response to the test nucleic acid molecule is stronger or weaker than a response to a reference nucleic acid molecule. The method involves the additional steps of comparing the response to a reference response when the TLR polypeptide is independently contacted with a reference nucleic acid molecule; and determining if the response is stronger or weaker than the reference response. In this embodiment the test nucleic acid molecule and the reference nucleic acid molecule are not able to compete or interact. For example, the reference response can be a parallel control or a historical control.

In another embodiment the method involves the additional steps of comparing the response to a reference response when the TLR polypeptide is concurrently contacted with a reference nucleic acid molecule; and determining if the response is stronger or weaker than the reference response. In this embodiment the test nucleic acid molecule and the reference nucleic acid molecule are potentially able to compete or interact since they are both present, for example, in a single reaction.

In another aspect the invention provides a screening method for identifying an immunostimulatory nucleic acid. The method according to this aspect involves contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a test nucleic acid molecule; detecting presence or absence of a response mediated by a TLR signal transduction pathway in the presence of the test nucleic acid molecule arising as a result of an interaction between the functional TLR and the test nucleic acid molecule; and determining the test nucleic acid molecule is an ISNA when the presence of a response mediated by the TLR signal transduction pathway is detected. A functional TLR refers to a TLR polypeptide or fragment thereof that can bind with a ligand and as a consequence of the binding engage at least one step or additional polypeptide in a TLR signal transduction pathway.

In one embodiment the method according to this aspect of the invention includes the further step of comparing (a) the response mediated by the TLR signal transduction pathway arising as a result of an interaction between the functional TLR and the test nucleic acid molecule with (b) a response arising as a result of an interaction between the functional TLR and a reference ISNA. In this and other screening assays of the instant invention, in preferred

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embodiments the screening method is performed on a plurality of test nucleic acids. In certain preferred embodiments the response mediated by the TLR signal transduction pathway is measured quantitatively, and the response mediated by the TLR signal transduction pathway associated with each of the plurality of test nucleic acid molecules is compared with 5 a response arising as a result of an interaction between the functional TLR and a reference ISNA.

In certain preferred embodiments a subset of the plurality of test nucleic acid molecules is selected based on the ability of the subset to produce a specific response mediated by the TLR signal transduction pathway. For example, the specific response can be 10 induction of a specific cytokine or panel of cytokines, e.g., Th1 cytokines, or, alternatively, inhibition of a specific cytokine or panel of cytokines, e.g., Th2 cytokines. The specific response can be induction, or, alternatively, inhibition of a specific class or subclass of antibody or panel of classes or subclasses of antibodies, e.g., Th1-associated antibodies or Th2-associated antibodies. The specific response in some embodiments can be activation or 15 inhibition of certain types of immune cells, e.g., B cells, dendritic cells (DCs), and natural killer (NK) cells. In some embodiments the specific response can be induction or inhibition of proliferation of certain types of immune cells, e.g., B cells, T cells, NK cells, dendritic cells, monocytes/macrophages. The subset of the plurality of test nucleic acids is therefore selected on the basis of the common association between the test nucleic acids of the subset 20 and the particular type of response mediated by the TLR signal transduction pathway. The particular type of response mediated by the TLR signal transduction pathway is typically, but not necessarily, an immune cell response.

In certain embodiments the response mediated by a TLR signal transduction pathway is selected from the group consisting of induction of a gene under control of NF- $\kappa$ B promoter 25 and secretion of a cytokine. In certain preferred embodiments the gene under control of NF- $\kappa$ B promoter is selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc. In certain preferred embodiments the cytokine is selected from the group consisting of IL-8, TNF- $\alpha$ , and IL-12 p40.

In certain preferred embodiments the reference ISNA is a CpG nucleic acid. 30 In certain preferred embodiments the test nucleic acid molecule is a CpG nucleic acid. According to this and other aspects of the invention involving functional TLR in a

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screening assay, in some embodiments the functional TLR is expressed in a cell. In some embodiments the functional TLR is naturally expressed by the cell. In certain preferred embodiments the cell is an isolated mammalian cell that naturally expresses the functional TLR. Whether the cell expresses the TLR naturally or the cell expresses the TLR because an expression vector having an isolated nucleic acid molecule encoding the TLR operatively linked to a promoter has been introduced into the cell, in some embodiments the cell further includes an expression vector comprising an isolated nucleic acid which encodes a reporter construct selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc, operatively linked to a promoter.

10 Also according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments the functional TLR is part of a cell-free system.

Also according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments the functional TLR is part of a complex with another TLR. In certain preferred embodiments the complex is a complex of TLR9 and TLR7. In certain preferred embodiments the complex is a complex of TLR9 and TLR8.

15 Also according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments the functional TLR is part of a complex with a non-TLR protein selected from the group consisting of MyD88, IRAK, TRAF6, I $\kappa$ B, NF- $\kappa$ B, and functional homologues and derivatives thereof.

20 Further according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments the response mediated by a TLR signal transduction pathway is selected from the group consisting of induction of a gene under control of NF- $\kappa$ B promoter and secretion of a cytokine.

Also according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments the gene under control of NF- $\kappa$ B promoter is selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc.

25 Also according to this and other aspects of the invention involving functional TLR in a screening assay, in certain embodiments wherein the cytokine is selected from the group consisting of IL-8, TNF- $\alpha$ , and IL-12 p40.

30 In a further aspect, the invention provides a screening method for comparing TLR

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signaling activity of a test compound with an ISNA. The method entails contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a reference ISNA and detecting a reference response mediated by a TLR signal transduction pathway; contacting a functional TLR selected from the group consisting of TLR7, TLR8, 5 and TLR9 with a test compound and detecting a test response mediated by a TLR signal transduction pathway; and comparing the test response with the reference response to compare the TLR signaling activity of the test compound with the ISNA.

In certain embodiments according to this aspect of the invention, the reference ISNA is a CpG nucleic acid.

10 In certain embodiments according to this aspect of the invention, the test compound is a polypeptide. In certain embodiments the test compound is part of a combinatorial library of compounds.

15 In certain embodiments the functional TLR is contacted with the reference ISNA and the test compound independently. Accordingly, in certain embodiments the screening method is a method for identifying an ISNA mimic, and the test compound is determined to be an ISNA mimic when the test response is similar to the reference response obtained with the reference ISNA. A test response is similar to the reference response when the test and reference responses are qualitatively alike, even if not quantitatively alike. Thus, for example, the test and reference responses are considered alike when both responses include 20 induction of a Th1-like immune response. The test response can be quantitatively less than, about the same as, or greater than the reference response.

25 In certain other embodiments the functional TLR is contacted with the reference ISNA and the test compound concurrently to produce a test-reference response mediated by a TLR signal transduction pathway, wherein the test-reference response may be compared to the reference response. In certain preferred embodiments the screening method is a method for identifying an ISNA agonist, wherein the test compound is an ISNA agonist when the test-reference response is greater than the reference response. In certain preferred embodiments the screening method is a method for identifying an ISNA antagonist, wherein the test compound is an ISNA antagonist when the test-reference response is less than the reference 30 response.

In a further aspect the invention provides a screening method for identifying species

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specificity of an ISNA. The method according to this aspect of the invention involves contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a first species with a test ISNA; contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a second species with the test ISNA; measuring a response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test ISNA; measuring a response mediated by the TLR signal transduction pathway associated with the contacting the functional TLR of the second species with the test ISNA; and comparing (a) the response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test ISNA with (b) the response mediated by the TLR signal transduction pathway associated with the contacting the functional TLR of the second species with the test ISNA. In preferred embodiments the TLR of the first species corresponds to the TLR of the second species, e.g., the TLR of the first species is human TLR9 and the TLR of the second species is murine TLR9. In certain embodiments the functional TLR may be expressed in a cell, part of a cell-free system, or part of a complex with another TLR or with a non-TLR protein, as previously described.

In yet another aspect the invention provides a method for identifying lead compounds for a pharmacological agent useful in the treatment of disease associated with TLR9 signaling activity. The method according to this aspect of the invention involves providing a cell comprising a TLR9 polypeptide or fragment thereof as provided in the second aspect of the invention; contacting the cell with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of TLR9 signaling activity; and determining a second amount of TLR9 signaling activity as a measure of the effect of the pharmacological agent on the TLR9 signaling activity, wherein a second amount of TLR9 signaling activity which is less than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces TLR9 signaling activity and wherein a second amount of TLR9 signaling activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases TLR9 signaling activity.

These and other aspects of the invention are described in greater detail below.

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#### Brief Description of the Figures

FIG. 1 is two paired bar graphs showing (A) the induction of NF- $\kappa$ B and (B) the amount of IL-8 produced by 293 fibroblast cells transfected with human TLR9 in response to exposure to various stimuli, including CpG-ODN, GpC-ODN, LPS, and medium.

5 FIG. 2 is a bar graph showing the induction of NF- $\kappa$ B produced by 293 fibroblast cells transfected with murine TLR9 in response to exposure to various stimuli, including CpG-ODN, methylated CpG-ODN (Me-CpG-ODN), GpC-ODN, LPS, and medium.

10 FIG. 3 is a series of gel images depicting the results of reverse transcriptase-polymerase chain reaction (RT-PCR) assays for murine TLR9 (mTLR9), human TLR9 (hTLR9), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) in untransfected control 293 cells, 293 cells transfected with mTLR9 (293-mTLR9), and 293 cells transfected with hTLR9 (293-hTLR9).

15 FIG. 4 is a graph showing the degree of induction of NF- $\kappa$ B-luc by various stimuli in stably transfected 293-hTLR9 cells.

FIG. 5 is a graph showing the degree of induction of NF- $\kappa$ B-luc by various stimuli in stably transfected 293-mTLR9 cells.

20 FIG. 6 is an image of a Coomassie-stained polyacrylamide gel depicting the presence of soluble hTLR9 in the supernatants of yeast cells transfected with hTLR9, either induced (lane 1) or not induced (lane 2).

FIG. 7 is a graph showing proliferation of human B cells in response to various stimuli, including *Escherichia coli* (*E. coli*) DNA, DNase-digested *E. coli* DNA, CpG-ODN, GpC-ODN, and LPS.

25 FIG. 8 is two paired bar graphs showing induction of (top) IL-8 and (bottom) TNF in plasmacytoid dendritic cells (CD123+ DC) and monocyte-derived dendritic cells (MDDC) in response to various stimuli, including *E. coli* DNA, DNase-digested *E. coli* DNA, CpG-ODN, GpC-ODN, and LPS.

30 FIG. 9 is a series of images of stained gels showing results of semi-quantitative RT-PCR comparing relative levels of human TLR9, TLR2, and TLR4 mRNA expression in human peripheral blood cells: MDDC (lane 1), purified CD14+ monocytes (lane 2), B cells (lane 3), CD123+ DC (lane 4), CD4+ T cells (lane 5), and CD8+ T cells (lane 6). GAPDH is a control for equalizing amounts of cDNA.

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FIG. 10 is a pair of graphs showing amounts of IL-12 induced in (A) human peripheral blood mononuclear cells (PBMC) and (B) murine splenocytes in response to shown concentrations of various ODN, including ODN 2006 (filled circles), 2006-GC (open circles), 1668 (filled triangles), and 1668-GC (open triangles).

5 FIG. 11 is a quartet of graphs depicting responsiveness of 293 cells transfected with hTLR9 (left panels) or mTLR9 (right panels) upon stimulation with shown concentrations of various ODN, including ODN 2006 (filled circles), 2006-GC (open circles), 1668 (filled triangles), and 1668-GC (open triangles). Responses are shown in terms of induction of NF- $\kappa$ B-luc (upper panels) and IL-8 (lower panels).

10 FIG. 12 is a bar graph depicting the dose-response of 293-hTLR9 cells to *E. coli* DNA (black bars) and to DNase-digested *E. coli* DNA (gray bars).

FIG. 13 is a pair of graphs showing the responsiveness of (A) 293-hTLR9 and (B) 293-mTLR9 cells to shown concentrations of phosphodiester versions of ODN 2006 (filled circles), 2006-GC (open circles), 1668 (filled triangles), and 1668-GC (open triangles).

15 Fig. 14 is a pair of graphs showing the responsiveness of 293-hTLR9 and 293-mTLR9 cells to shown concentrations of ODN 5002 (filled circles) and ODN 5007 (open circles).

FIG. 15 is a bar graph showing the response of 293 cells transfected with mTLR9 to CpG-ODN 1668 is inhibited in a dose-dependent manner by co-exposure to non-CpG-ODN PZ2.

20 FIG. 16 is a bar graph showing the response of 293-hTLR9 cells to CpG-ODN (black bars) or to TNF (gray bars) in the presence of shown amounts of blocking non-CpG-ODN.

FIG. 17 is a bar graph showing blockade of response of 293-hTLR9 cells to CpG-ODN, but not to IL-1 or TNF, in the presence of Bafilomycin A (gray bars). Control treatment with dimethyl sulfoxide (DMSO) is shown in black bars.

25 FIG. 18 is a graph showing the effect of varying concentrations of dominant negative human MyD88 on the induction of NF- $\kappa$ B in 293-hTLR9 cells stimulated with CpG-ODN (open circles), TNF- $\alpha$  (filled circles), or control (filled triangles).

FIG. 19 is a series of three Western blot images showing the response of various polyclonal antibodies to purified hTLR9-FLAG and mTLR9-FLAG: upper panel, anti-human and anti-mouse intracellular; middle, anti-mouse extracellular; and lower, anti-human extracellular. Arrows indicate position of TLR9 in each blot.

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FIG. 20 is a bar graph depicting the responsiveness of native form hTLR9 and hTLR9 variant form hTLR9-CXXCm to various stimuli at different concentrations.

FIG. 21 is a bar graph depicting the responsiveness of native form mTLR9 and mTLR9 variant form mTLR9-CXXCm to various stimuli at different concentrations.

5 FIG. 22 is a bar graph showing the responsiveness of native form mTLR9, mTLR9 variant form mTLR9-Phmut, and mTLR9 variant form mTLR9-MBDmut to various stimuli at different concentrations.

10 FIG. 23 is a bar graph showing the responsiveness of native form hTLR9, hTLR9 variant form hTLR9-PHmut, and hTLR9 variant form hTLR9-MBDmut to various stimuli at different concentrations.

FIG. 24 is a bar graph showing the responsiveness of native form mTLR9 and mTLR9 variant form mTLR9-TIRh to various stimuli at different concentrations.

FIG. 25 is a bar graph showing the responsiveness of native form hTLR9 and hTLR9 variant form hTLR9-TIRm to various stimuli at different concentrations.

15 FIG. 26 is a series of linear maps representing various features of human TLR7, TLR8, and TLR9 polypeptides.

FIG. 27 is an image of a silver stained polyacrylamide gel and schematic representation of a fusion protein in which the extracellular domain of human TLR9 (hTLR9) is fused to a human IgG1 Fc domain (hIgG-Fc) with a thrombin protease recognition site 20 interposed. From left to right, the gel was loaded with (1) supernatant of transfectants; (2) lysates of transfectants, treated with thrombin; (3) untreated lysates of transfectants; (4) molecular weight markers; (5) supernatant of mock transflectants; (6) lysates of mock 25 transflectants, treated with thrombin; and (7) untreated lysates of mock transflectants. Soluble hTLR9 and Fc are the products released from intact hTLR9-IgG-Fc following thrombin treatment. Molecular weights are indicated along the right side of the silver stain gel image.

#### Brief Description of Selected Sequences

SEQ ID NO:1 is the nucleotide sequence encoding a cDNA for murine TLR9.

SEQ ID NO:2 is the nucleotide sequence encoding the coding region of murine TLR9.

30 SEQ ID NO:3 is the amino acid sequence of a murine TLR9 encoded by SEQ ID NO:1.

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SEQ ID NO:173 is the nucleotide sequence encoding a cDNA for murine TLR7.

SEQ ID NO:174 is the nucleotide sequence encoding the coding region of murine TLR7.

SEQ ID NO:175 is the amino acid sequence of a murine TLR7 encoded by SEQ ID  
5 NO:173.

SEQ ID NO:190 is the nucleotide sequence encoding a cDNA for murine TLR8.

SEQ ID NO:191 is the nucleotide sequence encoding the coding region of murine TLR8.

SEQ ID NO:192 is the amino acid sequence of a murine TLR8 encoded by SEQ ID  
10 NO:190.

#### Detailed Description of the Invention

The present invention in one aspect involves the identification of cDNAs encoding mouse TLR9, referred to herein as murine TLR9 and, equivalently, mTLR9. The nucleotide sequence of the cDNA for murine TLR9 is presented as SEQ ID NO:1, the coding region of the cDNA for murine TLR9 is presented as SEQ ID NO:2, and the amino acid sequence of the murine TLR9 is presented as SEQ ID NO:3. The closely related human TLR9 (equivalently, hTLR9) was deposited in GenBank under accession numbers AF245704 and NM\_017742.

20 The nucleotide sequence of the cDNA for murine TLR9 presented as SEQ ID NO:1 is 3200 nucleotides long and includes the open reading frame (ORF, bases 40-3135) presented as SEQ ID NO:2 which spans 3096 nucleotides (excluding the stop codon). The amino acid sequence of the murine TLR9 presented as SEQ ID NO:3 is 1032 amino acids (aa) long, and it is believed to include an extracellular domain (aa 1-819), a transmembrane domain (aa 820-25 837), and an intracellular domain (aa 838-1032).

20 The amino acid sequence of human TLR9 (SEQ ID NO:6) and the amino acid sequence of the murine TLR9 (SEQ ID NO:3) are thus both 1032 amino acids long. Comparison of the aligned amino acid sequences for the murine and the human TLR9 molecules reveals a single base insertion at aa 435 of the murine TLR9 and a single base deletion at aa 860 of the human TLR9. (See Table 4 below.)

Whereas much of the polypeptide presented herein is identical to human TLR9,

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murine TLR9 has several single amino acid differences. These differences in amino acids are specifically amino acids 2, 3, 4, 6, 7, 18, 19, 22, 38, 44, 55, 58, 61, 62, 63, 65, 67, 71, 80, 84, 87, 88, 91, 101, 106, 109, 117, 122, 123, 134, 136, 140, 143, 146, 147, 157, 160, 161, 167, 168, 171, 185, 186, 188, 189, 191, 199, 213, 217, 220, 227, 231, 236, 245, 266, 269, 270, 5 271, 272, 273, 274, 278, 281, 285, 297, 298, 301, 305, 308, 311, 322, 323, 325, 326, 328, 332, 335, 346, 348, 353, 355, 358, 361, 362, 365, 367, 370, 372, 380, 381, 382, 386, 389, 392, 394, 397, 409, 412, 413, 415, 416, 419, 430, 432, 434, 435, 438, 439, 443, 444, 446, 447, 448, 450, 451, 452, 454, 455, 459, 460, 463, 465, 466, 468, 469, 470, 472, 473, 474, 475, 478, 488, 489, 494, 495, 498, 503, 508, 510, 523, 531, 539, 540, 543, 547, 549, 561, 10 563, 565, 576, 577, 579, 580, 587, 590, 591, 594, 595, 597, 599, 601, 603, 610, 611, 613, 616, 619, 632, 633, 640, 643, 645, 648, 650, 657, 658, 660, 667, 670, 672, 675, 679, 689, 697, 700, 703, 705, 706, 711, 715, 716, 718, 720, 723, 724, 726, 729, 731, 735, 737, 743, 749, 750, 751, 752, 754, 755, 759, 760, 772, 774, 780, 781, 786, 787, 788, 800, 814, 821, 829, 831, 832, 835, 844, 857, 858, 859, 862, 864, 865, 866, 879, 893, 894, 898, 902, 910, 15 917, 927, 949, 972, 975, 976, 994, 997, 1000, 1003, 1004, 1010, 1011, 1018, 1023, and 1027 of SEQ ID NO:3

In some forms the mouse protein mTLR9 contains a signal sequence at the N-terminus (amino acids 1-26) which allows transport to the endoplasmic reticulum and subsequently to the cell surface or intracellular compartments. A transmembrane region (amino acids 820-20 837) anchors the protein to the cell membrane. The cytoplasmic tail contains a Toll/IL-1 receptor (TIR) homology domain which is believed to function in signaling upon ligand binding. Leucine-rich-repeats (LRR) can be found in the extracellular region (a common feature of TLRs) and may be involved in ligand binding or dimerization of the molecule.

Both mouse and human TLR9 have an N-terminal extension of approximately 180 25 amino acids compared to other TLRs. An insertion also occurs at amino acids 253-268, which is not found in TLRs 1-6 but is present in human TLR7 and human TLR8. (See Figure 26.) This insert has two CXXC motifs which participate in forming a CXXC domain. The CXXC domain resembles a zinc finger motif and is found in DNA-binding proteins and in certain specific CpG binding proteins, e.g., methyl-CpG binding protein-1 (MBD-1). 30 Fujita N et al., *Mol Cell Biol* 20:5107-5118 (2000). Both human and mouse TLR9 CXXC domains occur at aa 253-268:

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CXXC motif:	GNCXXCXXXXXXCXXC	SEQ ID NO:196
Human TLR9:	GNCRRCDHAPNPCMEC	SEQ ID NO:197
Murine TLR9:	GNCRRCDHAPNPCMIC	SEQ ID NO:198

5

An additional motif involved in CpG binding is the MBD motif, also found in MBD-1, listed below as SEQ ID NO:125. Fujita, N et al., *Mol Cell Biol* 20:5107-18 (2000); Ohki I et al., *EMBO J* 18:6653-6661 (1999). Amino acids 524-554 of hTLR9 and aa 525-555 of mTLR9 correspond to the MBD motif of MBD-1 as shown:

10

MBD motif:

MBD-1	R-XXXXXXXX-R-X-D-X-Y-XXXXXXXXX-R-S-XXXXXX-Y	SEQ ID NO:125
hTLR9	Q-XXXXXXXX-K-X-D-X-Y-XXXXXXXXX-R-L-XXXXXX-Y	SEQ ID NO:126
mTLR9	Q-XXXXXXXX-K-X-D-X-Y-XXXXXXXXX-Q-L-XXXXXX-Y	SEQ ID NO:127
hTLR9	Q-VLDLSRN-K-L-D-L-Y-HEHSFTELP-R-L-EALDLS-Y	SEQ ID NO:210
mTLR9	Q-VLDLSHN-K-L-D-L-Y-HWKSFSELP-Q-L-QALDLS-Y	SEQ ID NO:211

15

Although the signaling functions of MBD-1 and TLR9 are quite different, the core D-X-Y is involved in CpG binding and is conserved. The C-terminal octamer S-XXXXXX-Y of the MBD motif may not be involved in binding and the S is not conserved by TLR9. The other mismatches are highly conserved or moderately conserved; example R to K or R to Q. These changes could explain MBD-1 as a methyl-CpG binder and TLR9 as a non-methyl-CpG binder. The differences between mouse and human TLR9 may explain inter-species differences in CpG-motif sequence selectivity. See **Figure 14** for inter-species sequence selectivity.

As discussed in Example 11 below and shown in **Figures 22 and 23**, the D-X-Y core of this MBD motif occurs as D-L-Y in both mTLR9 (aa 535-537) and hTLR9 (aa 534-536). Substitution of A for D and A for Y in the D-X-Y core, resulting in A-L-A in place of D-L-Y, destroys receptor activity for mTLR9 and hTLR9 alike.

The invention involves in one aspect murine TLR9 nucleic acids and polypeptides, as

well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing nucleic acids and polypeptides; complements of the foregoing nucleic acids; and molecules which selectively bind the foregoing nucleic acids and polypeptides.

5       The murine TLR9 nucleic acids and polypeptides of the invention are isolated. As used herein with respect to nucleic acids, the term "isolated" means: (i) amplified *in vitro* by, for example, polymerase chain reaction (PCR); (ii) recombinantly produced by cloning; (iii) purified, as by cleavage and gel separation; or (iv) synthesized by, for example, chemical synthesis. An isolated nucleic acid is one which is readily manipulable by recombinant DNA 10 techniques well known in the art. Thus, a nucleotide sequence contained in a vector in which 5' and 3' restriction sites are known or for which PCR primer sequences have been disclosed is considered isolated, but a nucleic acid sequence existing in its native state in its natural host is not. An isolated nucleic acid may be substantially purified, but need not be. For example, a nucleic acid that is isolated within a cloning or expression vector is not pure in that it may 15 comprise only a tiny percentage of the material in the cell in which it resides. Such a nucleic acid is isolated, however, as the term is used herein because it is readily manipulable by standard techniques known to those of ordinary skill in the art. An isolated nucleic acid as used herein is not a naturally occurring chromosome.

As used herein with respect to polypeptides, "isolated" means separated from its 20 native environment and present in sufficient quantity to permit its identification or use. Isolated, when referring to a protein or polypeptide, means, for example: (i) selectively produced by expression cloning or (ii) purified as by chromatography or electrophoresis. Isolated proteins or polypeptides may be, but need not be, substantially pure. The term "substantially pure" means that the proteins or polypeptides are essentially free of other 25 substances with which they may be found in nature or *in vivo* systems to an extent practical and appropriate for their intended use. Substantially pure polypeptides may be produced by techniques well known in the art. Because an isolated protein may be admixed with a pharmaceutically acceptable carrier in a pharmaceutical preparation, the protein may comprise only a small percentage by weight of the preparation. The protein is nonetheless 30 isolated in that it has been separated from the substances with which it may be associated in living systems, i.e., isolated from other proteins.

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As used herein a murine TLR9 nucleic acid refers to an isolated nucleic acid molecule which codes for a murine TLR9 polypeptide. Such nucleic acid molecules code for murine TLR9 polypeptides which include the sequence of SEQ ID NO:3 and fragments thereof. The nucleic acid molecules include the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:2, and 5 nucleotide sequences which differ from the sequences of SEQ ID NO:1 and SEQ ID NO:2 in codon sequence due to the degeneracy of the genetic code. The murine TLR9 nucleic acids of the invention also include alleles of the foregoing nucleic acids, as well as fragments of the foregoing nucleic acids. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction. Preferred murine TLR9 nucleic acids 10 include the nucleic acid sequence of SEQ ID NO:1 and SEQ ID NO:2. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein a murine TLR9 nucleic acid or murine TLR9 polypeptide also embraces homologues and alleles of murine TLR9. In general homologues and alleles typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity to 15 the sequences of specified nucleic acids and polypeptides, respectively. Thus homologues and alleles of murine TLR9 typically will share at least 40% nucleotide identity and/or at least 50% amino acid identity to the sequences of murine TLR9 nucleic acids and TLR9 polypeptides, respectively. In some instances homologues and alleles will share at least 50% nucleotide identity and/or at least 65% amino acid identity and in still other instances will 20 share at least 60% nucleotide identity and/or at least 75% amino acid identity. Preferably the homologues and alleles will share at least 80% nucleotide identity and/or at least 90% amino acid identity, and more preferably will share at least 90% nucleotide identity and/or at least 95% amino acid identity. Most preferably the homologues and alleles will share at least 95% nucleotide identity and/or at least 99% amino acid identity. The homology can be calculated 25 using various publicly available software tools developed by the National Center for Biotechnology Information (NCBI, Bethesda, Maryland) that can be obtained through the internet (<ftp://ncbi.nlm.nih.gov/pub/>). Exemplary tools include the BLAST system available from the NCBI at <http://www.ncbi.nlm.nih.gov>, used with default settings. Pairwise and ClustalW alignments (BLOSUM30 matrix setting) as well as Kyte-Doolittle hydropathic 30 analysis can be obtained, for example, using the MacVector sequence analysis software (Oxford Molecular Group). Watson-Crick complements of the foregoing nucleic acids also

are embraced by the invention.

Alleles of the murine TLR9 nucleic acids of the invention can be identified by conventional techniques. For example, alleles of murine TLR9 can be isolated by hybridizing a probe which includes at least a fragment of SEQ ID NO:1 or SEQ ID NO:2 under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for murine TLR9 polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:1 or SEQ ID NO:2 under stringent conditions.

In screening for murine TLR nucleic acids, a Southern blot may be performed using the foregoing stringent conditions, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal. Corresponding non-radioactive methods are also well known in the art and can be used to similar effect.

The murine TLR nucleic acids of the invention also include degenerate nucleic acids which include alternative codons to those present in the native materials. For example, serine residues are encoded by the codons AGC, AGT, and TCA, TCC, TCG and TCT. Each of the six codons is equivalent for the purposes of encoding a serine residue. Thus, it will be apparent to one of ordinary skill in the art that any of the serine-encoding nucleotide triplets may be employed to direct the protein synthesis apparatus, *in vitro* or *in vivo*, to incorporate a serine residue into an elongating murine TLR polypeptide. Similarly, nucleotide sequence triplets which encode other amino acid residues include, but are not limited to: CCA, CCC, CCG and CCT (proline codons); CGA, CGC, CGG, CGT, AGA and AGG (arginine codons); ACA, ACC, ACG and ACT (threonine codons); AAC and AAT (asparagine codons); and ATA, ATC and ATT (isoleucine codons). As is well known by those of ordinary skill in the art, other specific amino acid residues may be encoded similarly by multiple nucleotide sequences. Thus, the invention embraces degenerate nucleic acids that differ from the biologically isolated nucleic acids in codon sequence due to the degeneracy of the genetic code. The above-noted codon degeneracy notwithstanding, it is well appreciated by those skilled in the art that there are certain codon usage preferences in specific host organisms, such that in practice it may be preferred to select or to avoid certain degenerate codons in a particular host.

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The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. The modified nucleic acid molecules according to this aspect of the invention exclude fully native human TLR9 nucleic acid molecules (GenBank Accession No. AF245704 (SEQ ID NO:4) or GenBank Accession No.

5 NM\_017442 (SEQ ID NO:5)). In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or the polypeptides, such as signaling activity, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are described  
10 elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

For example, modified nucleic acid molecules which encode polypeptides having  
15 single amino acid changes can be prepared. Each of these nucleic acid molecules can have one, two or three nucleotide substitutions exclusive of nucleotide changes corresponding to the degeneracy of the genetic code as described herein. Likewise, modified nucleic acid molecules which encode polypeptides having two amino acid changes can be prepared which have, e.g., 2-6 nucleotide changes. Numerous modified nucleic acid molecules like these will  
20 be readily envisioned by one of skill in the art, including for example, substitutions of nucleotides in codons encoding amino acids 2 and 3, 2 and 4, 2 and 5, 2 and 6, and so on. In the foregoing example, each combination of two amino acids is included in the set of modified nucleic acid molecules, as well as all nucleotide substitutions which code for the  
25 amino acid substitutions. Additional nucleic acid molecules that encode polypeptides having additional substitutions (i.e., 3 or more), additions or deletions (e.g., by introduction of a stop codon or a splice site(s)) also can be prepared and are embraced by the invention as readily envisioned by one of ordinary skill in the art. Any of the foregoing nucleic acids or polypeptides can be tested by routine experimentation for retention of structural relation or activity to the nucleic acids and/or polypeptides disclosed herein.

30 The invention also provides isolated fragments of SEQ ID NO:1 and SEQ ID NO:2. The fragments can be used as probes in Southern blot assays to identify such nucleic acids, or

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they can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween, and are useful, e.g., as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 200, 250, 300, 400 or 5 more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the murine TLR9 10 polypeptides, useful, for example, in the preparation of antibodies, in immunoassays, and the like. The foregoing nucleic acid fragments further can be used as antisense molecules to inhibit the expression of murine TLR9 nucleic acids and polypeptides, particularly for therapeutic purposes as described in greater detail below.

The invention also includes functionally equivalent variants of the murine TLR9, which include variant nucleic acids and polypeptides which retain one or more of the 15 functional properties of the murine TLR9. Preferably such variants include the murine-specific N-terminal domain (e.g., amino acids 1-819 or amino acids 1-837 of SEQ ID NO:3). For example, variants include a fusion protein which includes the extracellular and transmembrane domains of the murine TLR9 (i.e., amino acids 1-837) which retains the ability to interact with extracellular molecules in a manner similar to intact murine TLR9. 20 Alternative variants include, for example, a fusion protein which includes the cytoplasmic domain of murine TLR9 (i.e., amino acids 838-1032) which retains the ability to interact with intracellular molecules in a manner similar to intact murine TLR9. Still other functionally equivalent variants include truncations, deletions, point mutations, or additions of amino acids to the sequence of SEQ ID NO:3 which retain functions of SEQ ID NO:3. For example, 25 the FLAG peptide sequence (DYKDDDDK) can be added at the N-terminal end, or green fluorescent protein (GFP) can be added at the C-terminal end. All such addition variant polypeptides are preferably made by translation of modified nucleic acids based on SEQ ID NO:1 or SEQ ID NO:2 with corresponding appropriate coding nucleic acid sequence appended thereto with maintenance of the proper reading frame.

30 Functionally equivalent variants also include a murine TLR9 which has had a portion (e.g., of the N-terminus) removed or replaced by a similar domain from another TLR (e.g., a

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"domain-swapping" variant). Examples of such domain-swapping variants include at least two types: those involving swapping a TLR9 domain from one species with a TLR9 domain from another species, and those involving swapping a TLR domain from TLR9 with a TLR domain from another TLR. In certain preferred embodiments the swapping involves

5 corresponding domains between the different TLR molecules. It is believed that certain such domain-swapping variants are not functionally equivalent in a literal sense, insofar as they can function in a manner superior to either or both intact parent TLR molecules from which a particular domain-swapping variant derives. For example, the TLR/IL-1R signaling mediated by human TLR9 could be limited, not by the capacity of its extracellular domain to interact

10 with CpG ODN, but rather by the capacity of its cytoplasmic domain to engage the TLR/IL-1R signaling pathway. In such a circumstance, it could be advantageous to substitute a more potent cytoplasmic domain from a TLR9 from another species. Such a domain-swapping variant, e.g., chimeric hTLR9/mTLR9, could be used in screening assays for CpG immuno-agonist/antagonists to increase the sensitivity of the assay, without changing the species

15 specificity.

Other functionally equivalent variants will be known to one of ordinary skill in the art, as will be methods for preparing such variants. The activity of a functionally equivalent variant can be determined using the methods provided herein, and in references that have described assays using other TLRs and TLRs of other species. Such variants are useful, *inter alia*, for evaluating bioavailability of drugs, in assays for identification of compounds which bind to and/or regulate the signaling function of the murine TLR9, and for determining the portions of the murine TLR9 which are required for signaling activity.

Variants which are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing TLR9 signaling activity. Examples of non-functional variants include those incorporating a mutation of proline at aa 915 to histidine (P915H) which renders both mTLR9 and hTLR9 nonfunctional with respect to signaling. Further examples of non-functional variants include those incorporating a mutation of the D-X-Y core of the MBD motif to A-L-A, as discussed above, to render both mTLR9 and hTLR9 nonfunctional with respect to CpG DNA binding.

30 A murine TLR9 nucleic acid, in one embodiment, is operably linked to a gene expression sequence which can direct the expression of the murine TLR9 nucleic acid within

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a eukaryotic or prokaryotic cell. A "gene expression sequence" is any regulatory nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the nucleic acid to which it is operably linked.

With respect to murine TLR9 nucleic acid, the "gene expression sequence" is any regulatory

- 5 nucleotide sequence, such as a promoter sequence or promoter-enhancer combination, which facilitates the efficient transcription and translation of the murine TLR9 nucleic acid to which it is operably linked. The gene expression sequence may, for example, be a mammalian or viral promoter, such as a constitutive or inducible promoter. Constitutive mammalian promoters include, but are not limited to, the promoters for the following genes: hypoxanthine  
10 phosphoribosyl transferase (HPRT), adenosine deaminase, pyruvate kinase,  $\beta$ -actin promoter, and other constitutive promoters. Exemplary viral promoters which function constitutively in eukaryotic cells include, for example, promoters from the simian virus (e.g., SV40), papilloma virus, adenovirus, human immunodeficiency virus (HIV), Rous sarcoma virus (RSV), cytomegalovirus (CMV), the long terminal repeats (LTR) of Moloney murine  
15 leukemia virus and other retroviruses, and the thymidine kinase (TK) promoter of herpes simplex virus. Other constitutive promoters are known to those of ordinary skill in the art. The promoters useful as gene expression sequences of the invention also include inducible promoters. Inducible promoters are expressed in the presence of an inducing agent. For example, the metallothionein (MT) promoter is induced to promote transcription and  
20 translation in the presence of certain metal ions. Other inducible promoters are known to those of ordinary skill in the art.

In general, the gene expression sequence shall include, as necessary, 5' non-transcribing and 5' non-translating sequences involved with the initiation of transcription and translation, respectively, such as a TATA box, capping sequence, CAAT sequence, and the like. Especially, such 5' non-transcribing sequences will include a promoter region which includes a promoter sequence for transcriptional control of the operably joined murine TLR9 nucleic acid. The gene expression sequences optionally include enhancer sequences or upstream activator sequences as desired.

Generally a nucleic acid coding sequence and a gene expression sequence are said to

- 30 be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the nucleic acid coding sequence under the influence or

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control of the gene expression sequence. Thus the murine TLR9 nucleic acid sequence and the gene expression sequence are said to be "operably linked" when they are covalently linked in such a way as to place the transcription and/or translation of the murine TLR9 coding sequence under the influence or control of the gene expression sequence. If it is desired that

- 5 the murine TLR9 sequence be translated into a functional protein, two DNA sequences are said to be operably linked if induction of a promoter in the 5' gene expression sequence results in the transcription of the murine TLR9 sequence and if the nature of the linkage between the two DNA sequences does not (1) result in the introduction of a frame-shift mutation, (2) interfere with the ability of the promoter region to direct the transcription of the  
10 murine TLR9 sequence, or (3) interfere with the ability of the corresponding RNA transcript to be translated into a protein. Thus, a gene expression sequence would be operably linked to a murine TLR9 nucleic acid sequence if the gene expression sequence were capable of effecting transcription of that murine TLR9 nucleic acid sequence such that the resulting transcript might be translated into the desired protein or polypeptide.

15 The murine TLR9 nucleic acid molecules and the murine TLR9 polypeptides (including the murine TLR9 inhibitors described below) of the invention can be delivered to the eukaryotic or prokaryotic cell alone or in association with a vector. In its broadest sense, a "vector" is any vehicle capable of facilitating: (1) delivery of a nucleic acid or polypeptide to a target cell, (2) uptake of a nucleic acid or polypeptide by a target cell, or (3) expression of  
20 a nucleic acid molecule or polypeptide in a target cell. In this particular setting, a "vector" is any vehicle capable of facilitating: (1) delivery of a murine TLR9 nucleic acid or polypeptide to a target cell, (2) uptake of a murine TLR9 nucleic acid or polypeptide by a target cell, or (3) expression of a murine TLR9 nucleic acid molecule or polypeptide in a target cell.

Preferably, the vectors transport the murine TLR9 nucleic acid or polypeptide into the target

- 25 cell with reduced degradation relative to the extent of degradation that would result in the absence of the vector. Optionally, a "targeting ligand" can be attached to the vector to selectively deliver the vector to a cell which expresses on its surface the cognate receptor (e.g., a receptor, an antigen recognized by an antibody) for the targeting ligand. In this manner, the vector (containing a murine TLR9 nucleic acid or a murine TLR9 polypeptide)  
30 can be selectively delivered to a specific cell. In general, the vectors useful in the invention are divided into two classes: biological vectors and chemical/physical vectors. Biological

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vectors are more useful for delivery/uptake of murine TLR9 nucleic acids to/by a target cell. Chemical/physical vectors are more useful for delivery/uptake of murine TLR9 nucleic acids or murine TLR9 proteins to/by a target cell.

Biological vectors include, but are not limited to, plasmids, phagemids, viruses, other vehicles derived from viral or bacterial sources that have been manipulated by the insertion or incorporation of the nucleic acid sequences of the invention, and free nucleic acid fragments which can be linked to the nucleic acid sequences of the invention. Viral vectors are a preferred type of biological vector and include, but are not limited to, nucleic acid sequences from the following viruses: retroviruses, such as Moloney murine leukemia virus; Harvey murine sarcoma virus; murine mammary tumor virus; Rous sarcoma virus; adenovirus; adeno-associated virus; SV40-type viruses; polyoma viruses; poxviruses; Epstein-Barr viruses; papilloma viruses; herpes virus; vaccinia virus; and polio virus. One can readily employ other vectors not named but known in the art.

Preferred viral vectors are based on non-cytopathic eukaryotic viruses in which non-essential genes have been replaced with the gene of interest. Non-cytopathic viruses include retroviruses, the life cycle of which involves reverse transcription of genomic viral RNA into DNA with subsequent proviral integration into host cellular DNA. In general, the retroviruses are replication-deficient (i.e., capable of directing synthesis of the desired proteins, but incapable of manufacturing an infectious particle). Such genetically altered retroviral expression vectors have general utility for the high-efficiency transduction of genes *in vivo*. Standard protocols for producing replication-deficient retroviruses (including the steps of incorporation of exogenous genetic material into a plasmid, transfection of a packaging cell line with plasmid, production of recombinant retroviruses by the packaging cell line, collection of viral particles from tissue culture media, and infection of the target cells with viral particles) are provided in Kriegler, M., "Gene Transfer and Expression, A Laboratory Manual," W.H. Freeman Co., New York (1990) and Murray, E.J., ed., "Methods in Molecular Biology," vol. 7, Humana Press, Inc., Clifton, New Jersey (1991).

Another preferred virus for certain applications is the adeno-associated virus (AAV), a double-stranded DNA virus. The AAV can be engineered to be replication-deficient and is capable of infecting a wide range of cell types and species. It further has advantages, such as heat and lipid solvent stability; high transduction frequencies in cells of diverse lineages; and

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lack of superinfection inhibition thus allowing multiple series of transductions. Reportedly, the AAV can integrate into human cellular DNA in a site-specific manner, thereby minimizing the possibility of insertional mutagenesis and variability of inserted gene expression. In addition, wild-type AAV infections have been followed in tissue culture for greater than 100 passages in the absence of selective pressure, implying that the AAV genomic integration is a relatively stable event. The AAV can also function in an extrachromosomal fashion.

5 Expression vectors containing all the necessary elements for expression are commercially available and known to those skilled in the art. See, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. Cells are genetically engineered by the introduction into the cells of heterologous DNA (RNA) encoding a murine TLR9 polypeptide or fragment or variant thereof. That heterologous DNA (RNA) is placed under operable control of transcriptional elements to permit the expression of the heterologous 10 DNA in the host cell.

15 Preferred systems for mRNA expression in mammalian cells are those such as pRc/CMV (available from Invitrogen, Carlsbad, CA) that contain a selectable marker such as a gene that confers G418 resistance (which facilitates the selection of stably transfected cell lines) and the human CMV enhancer-promoter sequences. Additionally, suitable for expression in primate or canine cell lines is the pCEP4 vector (Invitrogen), which contains an Epstein Barr virus (EBV) origin of replication, facilitating the maintenance of plasmid as a multicopy extrachromosomal element. Another expression vector is the pEF-BOS plasmid containing the promoter of polypeptide Elongation Factor 1 $\alpha$ , which stimulates efficiently transcription *in vitro*. The plasmid is described by Mishizuma and Nagata (*Nucleic Acids Res* 20:5322 (1990)), and its use in transfection experiments is disclosed by, for example, Demoulin (*Mol Cell Biol* 16:4710-4716 (1996)). Still another preferred expression vector is an adenovirus, described by Stratford-Perricaudet, which is defective for E1 and E3 proteins (*J Clin Invest* 90:626-630 (1992)).

20 In addition to the biological vectors, chemical/physical vectors may be used to deliver a nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived

from bacteriological or viral sources, capable of delivering an isolated nucleic acid or polypeptide to a cell. As used herein with respect to a murine TLR9 nucleic acid or polypeptide, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated murine  
5 TLR9 nucleic acid or polypeptide to a cell.

A preferred chemical/physical vector of the invention is a colloidal dispersion system. Colloidal dispersion systems include lipid-based systems including oil-in-water emulsions, micelles, mixed micelles, and liposomes. A preferred colloidal system of the invention is a liposome. Liposomes are artificial membrane vesicles which are useful as a delivery vector *in vivo* or *in vitro*. It has been shown that large unilamellar vesicles (LUV), which range in size from 0.2 - 4.0  $\mu\text{m}$  can encapsulate large macromolecules. RNA, DNA, and intact virions can be encapsulated within the aqueous interior and be delivered to cells in a biologically active form (Fraley et al., *Trends Biochem Sci* 6:77 (1981)). In order for a liposome to be an efficient nucleic acid transfer vector, one or more of the following characteristics should be  
10 present: (1) encapsulation of the nucleic acid of interest at high efficiency with retention of biological activity; (2) preferential and substantial binding to a target cell in comparison to non-target cells; (3) delivery of the aqueous contents of the vesicle to the target cell cytoplasm at high efficiency; and (4) accurate and effective expression of genetic information.  
15

Liposomes may be targeted to a particular tissue by coupling the liposome to a specific ligand such as a monoclonal antibody, sugar, glycolipid, or protein. Ligands which may be useful for targeting a liposome to a particular cell will depend on the particular cell or tissue type. Additionally when the vector encapsulates a nucleic acid, the vector may be coupled to a nuclear targeting peptide, which will direct the murine TLR9 nucleic acid to the nucleus of the host cell.  
20

25 Liposomes are commercially available from Gibco BRL, for example, as LIPOFECTIN<sup>TM</sup> and LIPOFECTACE<sup>TM</sup>, which are formed of cationic lipids such as N-[1-(2, 3 dioleyloxy)-propyl]-N, N, N-trimethylammonium chloride (DOTMA) and dimethyl dioctadecylammonium bromide (DDAB). Methods for making liposomes are well known in the art and have been described in many publications.

30 Other exemplary compositions that can be used to facilitate uptake by a target cell of nucleic acids in general, and nucleic acids encoding the murine TLR9 in particular, include

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calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a murine TLR9 nucleic acid into a preselected location within a target cell chromosome).

5       The invention also embraces so-called expression kits, which allow the artisan to prepare a desired expression vector or vectors. Such expression kits include at least separate portions of the previously discussed coding sequences. Other components may be added, as desired, as long as the previously mentioned sequences, which are required, are included.

It will also be recognized that the invention embraces the use of the murine TLR9  
10 cDNA sequences in expression vectors to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., 293 fibroblast cells (ATCC, CRL-1573), MonoMac-6, THP-1, U927, CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as  
15 human, pig, goat, primate, rodent, guinea pig, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated murine TLR9 polypeptides which include the amino acid sequences of SEQ ID NO:3 and fragments thereof, encoded by the murine TLR9 nucleic acids described above. Murine TLR9 polypeptides also embrace alleles, functionally equivalent variants and analogs (those non-allelic polypeptides which vary in amino acid sequence from the disclosed murine TLR9 polypeptides by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides retain TLR9 activity. Non-functional variants also are embraced by the invention; these are useful as antagonists of TLR9 signaling function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes including as a component of assays.

Fragments of a polypeptide preferably are those fragments which retain a distinct functional capability of the intact polypeptide, in particular as a receptor of various molecules. Accordingly, fragments of a TLR9 polypeptide preferably are those fragments which retain a distinct functional capability of the TLR9 polypeptide, in particular as a receptor of various molecules. Of particular interest are fragments that bind to ISNAs, including, for example,

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fragments that bind CpG nucleic acids. Other functional capabilities which can be retained in a fragment of a polypeptide include signal transduction (e.g., TLR/IL-1R signaling by murine TLR9), interaction with antibodies and interaction with other polypeptides (such as would be found in a protein complex). Those skilled in the art are well versed in methods that can be

5 applied for selecting fragments which retain a functional capability of the murine TLR9.

Confirmation of the functional capability of the fragment can be carried out by synthesis of the fragment and testing of the capability according to standard methods. For example, to test the signaling activity of a murine TLR9 fragment, one inserts or expresses the fragment in a cell in which signaling can be measured. Such methods, which are standard in the art, are

10 described further herein.

The invention embraces variants of the murine TLR9 polypeptides described above.

As used herein, a "variant" of a polypeptide is a polypeptide which contains one or more modifications to the primary amino acid sequence of a polypeptide. Accordingly, a "variant" of a murine TLR9 polypeptide is a polypeptide which contains one or more modifications to

15 the primary amino acid sequence of a murine TLR9 polypeptide. Modifications which create a murine TLR9 variant can be made to a murine TLR9 polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a murine TLR9 polypeptide, such as

signaling; 2) to enhance a property of a murine TLR9 polypeptide, such as signaling, binding affinity for nucleic acid ligand or other ligand molecule, protein stability in an expression

20 system, or the stability of protein-protein binding; 3) to provide a novel activity or property to a murine TLR9 polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety, e.g., luciferase, FLAG peptide, GFP; 4) to establish that an amino acid

substitution does or does not affect molecular signaling activity; or 5) reduce immunogenicity of a murine TLR9 polypeptide. Modifications to a murine TLR9 polypeptide are typically

25 made to the nucleic acid which encodes the murine TLR9 polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties. Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety (for example, biotin, fluorophore, radioisotope, enzyme, or peptide), addition of a fatty acid, and the like.

30 Modifications also embrace fusion proteins comprising all or part of the murine TLR9

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amino acid sequence. One of skill in the art will be familiar with methods for predicting the effect on protein conformation of a change in protein sequence, and can thus "design" a variant murine TLR9 according to known methods. One example of such a method is described by Dahiyat and Mayo in *Science* 278:82-87 (1997), whereby proteins can be 5 designed *de novo*. The method can be applied to a known protein to vary only a portion of the polypeptide sequence. By applying the computational methods of Dahiyat and Mayo, specific variants of a murine TLR9 polypeptide can be proposed and tested to determine whether the variant retains a desired conformation.

Variants include murine TLR9 polypeptides which are modified specifically to alter a 10 feature of the polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a murine TLR9 polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

15 Mutations of a nucleic acid which encode a murine TLR9 polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide.

Mutations can be made by selecting an amino acid substitution, or by random 20 mutagenesis of a selected site in a nucleic acid which encodes the polypeptide. Variant polypeptides are then expressed and tested for one or more activities to determine which mutation provides a variant polypeptide with a desired property. Further mutations can be made to variants (or to non-variant murine TLR9 polypeptides) which are silent as to the amino acid sequence of the polypeptide, but which provide preferred codons for translation in 25 a particular host. The preferred codons for translation of a nucleic acid in, e.g., *E. coli*, are well known to those of ordinary skill in the art. Still other mutations can be made to the noncoding sequences of a murine TLR9 gene or cDNA clone to enhance expression of the polypeptide.

The activity of variants of murine TLR9 polypeptides can be tested by cloning the 30 gene encoding the variant murine TLR9 polypeptide into a prokaryotic or eukaryotic (e.g., mammalian) expression vector, introducing the vector into an appropriate host cell,

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expressing the variant murine TLR9 polypeptide, and testing for a functional capability of the murine TLR9 polypeptides as disclosed herein. For example, the variant murine TLR9 polypeptide can be tested for ability to provide signaling, as set forth below in the examples. Preparation of other variant polypeptides may favor testing of other activities, as will be known to one of ordinary skill in the art.

The skilled artisan will also realize that conservative amino acid substitutions may be made in murine TLR9 polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the murine TLR9 polypeptides. As used herein, a "conservative amino acid substitution" refers to an amino acid substitution which does not alter the relative charge or size characteristics of the polypeptide in which the amino acid substitution is made. Variants can be prepared according to methods for altering polypeptide sequence known to one of ordinary skill in the art such as are found in references which compile such methods, e.g., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989, or *Current Protocols in Molecular Biology*, F.M. Ausubel, et al., eds., John Wiley & Sons, Inc., New York. Exemplary functionally equivalent variants of the murine TLR9 polypeptides include conservative amino acid substitutions of SEQ ID NO:3. Conservative substitutions of amino acids include substitutions made amongst amino acids within the following groups: (a) M, I, L, V; (b) F, Y, W; (c) K, R, H; (d) A, G; (e) S, T; (f) Q, N; and (g) E, D.

Conservative amino acid substitutions in the amino acid sequence of murine TLR9 polypeptide to produce functionally equivalent variants of murine TLR9 typically are made by alteration of the nucleic acid sequence encoding murine TLR9 polypeptides (e.g., SEQ ID NO:1 and SEQ ID NO:2). Such substitutions can be made by a variety of methods known to one of ordinary skill in the art. For example, amino acid substitutions may be made by PCR-directed mutation, site-directed mutagenesis according to the method of Kunkel (Kunkel, *Proc Natl Acad Sci USA* 82:488-492 (1985)), or by chemical synthesis of a gene encoding a murine TLR9 polypeptide. The activity of functionally equivalent fragments of murine TLR9 polypeptides can be tested by cloning the gene encoding the altered murine TLR9 polypeptide into a bacterial or mammalian expression vector, introducing the vector into an appropriate host cell, expressing the altered murine TLR9 polypeptide, and testing for the ability of the

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murine TLR9 polypeptide to mediate a signaling event. Peptides which are chemically synthesized can be tested directly for function.

A variety of methodologies well known to the skilled practitioner can be utilized to obtain isolated murine TLR9 polypeptide molecules. The polypeptide may be purified from 5 cells which naturally produce the polypeptide by chromatographic means or immunological recognition. Alternatively, an expression vector may be introduced into cells to cause production of the polypeptide. In another method, mRNA transcripts may be microinjected or otherwise introduced into cells to cause production of the encoded polypeptide. Translation of mRNA in cell-free extracts such as the reticulocyte lysate system also may be used to 10 produce polypeptide. Those skilled in the art also can readily follow known methods for isolating murine TLR9 polypeptides. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography and immune-affinity chromatography.

The invention as described herein has a number of uses, some of which are described 15 elsewhere herein. For example, the invention permits isolation of the murine TLR9 polypeptide molecules by, e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another example, the isolation of the murine TLR9 gene makes it possible for murine TLR9 to be used in methods for assaying molecular interactions involving TLR9.

As discussed further in the Examples below, it has been discovered according to one 20 aspect of the invention that responsiveness to ISNA can be reconstituted in ISNA-unresponsive cells by introducing into ISNA-unresponsive cells an expression vector that directs the expression of murine TLR9 (and certain homologues and variants thereof). Cells so reconstituted also exhibit responses to substances other than phosphorothioate ISNA, e.g., 25 *E. coli* DNA, phosphodiester CpG-ODN, and even methylated CpG-ODN.

Also as discussed further in the Examples below, it has been discovered according to certain aspects of the instant invention that TLR9 not only confers upon cells the ability to signal in response to binding ISNA, but also confers both sequence specificity and species specificity to such signaling responses. Thus murine TLR9 signaling in response to CpG- 30 ODN 1668, reportedly an optimal murine ISNA, was found to be significantly stronger than the corresponding murine TLR9 signaling response to CpG-ODN 2006, reportedly an optimal

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human ISNA. The converse was also found to be true, i.e., human TLR9 signaling in response to CpG-ODN 2006 was found to be significantly stronger than the corresponding human TLR9 signaling response to CpG-ODN 1668. Furthermore, it has been discovered according to the instant invention that certain types of cells preferentially express TLR9. For example, TLR9 is strongly expressed in B cells and plasmacytoid dendritic cells (CD123+ DC), but only weakly by T cells, monocyte-derived dendritic cells (MDDC), and CD14+ monocytes. In contrast, TLR2 and TLR4 are strongly expressed by MDDC and CD14+ monocytes, but relatively weakly by B cells, CD123+ DC, and T cells.

The invention also embraces agents which bind selectively to the murine TLR9 nucleic acid molecules or polypeptides as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. The agents include polypeptides which bind to murine TLR9, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase murine TLR9-mediated signaling activity (antagonists and agonists, respectively).

Some of the agents are inhibitors. A murine TLR9 inhibitor is an agent that inhibits murine TLR9-mediated signaling across a cell membrane.

As used herein “TLR9 signaling” refers to an ability of a TLR9 polypeptide to activate the TLR/IL-1R (TIR) signaling pathway, also referred to herein as the TLR signal transduction pathway. Without meaning to be held to any particular theory, it is believed that the TLR/IL-1R signaling pathway involves signaling via the molecules myeloid differentiation marker 88 (MyD88) and tumor necrosis factor (TNF) receptor-associated factor 6 (TRAF6), leading to activation of kinases of the I $\kappa$ B kinase complex and the c-jun NH<sub>2</sub>-terminal kinases (e.g., JNK 1/2). Häcker H et al., *J Exp Med* 192:595-600 (2000). A molecule which inhibits TLR9 activity (an antagonist) is one which inhibits TLR9-mediated activation of the TLR/IL-1R signaling pathway, and a molecule which increases TLR9 signaling (an agonist) is one which increases TLR9-mediated activation of the TLR/IL-1R signaling pathway. Changes in TLR9 activity can be measured by assays such as those disclosed herein, including expression of genes under control of  $\kappa$ B-sensitive promoters and enhancers. Such naturally occurring genes include the genes encoding IL-1 $\beta$ , IL-6, IL-8, the p40 subunit of interleukin 12 (IL-12p40), and the costimulatory molecules CD80 and CD86. Other genes can be placed under the control of such regulatory elements (see below) and thus

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serve to report the level of TLR9 signaling. Additional nucleotide sequence can be added to SEQ ID NO:1 or SEQ ID NO:2, preferably to the 5' or the 3' end of SEQ ID NO:2, to yield a nucleotide sequence encoding a chimeric polypeptide that includes a detectable or reporter moiety, e.g., FLAG, luciferase (luc), green fluorescent protein (GFP) and others known by  
5 those skilled in the art. These are discussed in greater detail in the Examples below.

In one embodiment the murine TLR9 inhibitor is an antisense oligonucleotide that selectively binds to a murine TLR9 nucleic acid molecule, to reduce the expression of murine TLR9 (or TLR9 of another species) in a cell. This is desirable in virtually any medical condition wherein a reduction of TLR9 signaling activity is desirable.

10 As used herein, the term "antisense oligonucleotide" or "antisense" describes an oligonucleotide that is an oligoribonucleotide, oligodeoxyribonucleotide, modified oligoribonucleotide, or modified oligodeoxyribonucleotide which hybridizes under physiological conditions to DNA comprising a particular gene or to an mRNA transcript of that gene and, thereby, inhibits the transcription of that gene and/or the translation of that  
15 mRNA. The antisense molecules are designed so as to interfere with transcription or translation of a target gene upon hybridization with the target gene or transcript. Those skilled in the art will recognize that the exact length of the antisense oligonucleotide and its degree of complementarity with its target will depend upon the specific target selected, including the sequence of the target and the particular bases which comprise that sequence. It  
20 is preferred that the antisense oligonucleotide be constructed and arranged so as to bind selectively with the target under physiological conditions, i.e., to hybridize substantially more to the target sequence than to any other sequence in the target cell under physiological conditions.

Based upon SEQ ID NO:1 and SEQ ID NO:2, or upon allelic or homologous genomic  
25 and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention. In order to be sufficiently selective and potent for inhibition, such antisense oligonucleotides should comprise at least 10 and, more preferably, at least 15 consecutive bases which are complementary to the target, although in certain cases modified oligonucleotides as short as 7  
30 bases in length have been used successfully as antisense oligonucleotides. Wagner RW et al., *Nat Biotechnol* 14:840-844 (1996). Most preferably, the antisense oligonucleotides comprise

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a complementary sequence of 20-30 bases. Although oligonucleotides may be chosen which are antisense to any region of the gene or mRNA transcripts, in preferred embodiments the antisense oligonucleotides correspond to N-terminal or 5' upstream sites such as translation initiation, transcription initiation or promoter sites. In addition, 3'-untranslated regions may 5 be targeted. Targeting to mRNA splicing sites has also been used in the art but may be less preferred if alternative mRNA splicing occurs. In addition, the antisense is targeted, preferably, to sites in which mRNA secondary structure is not expected (see, e.g., Sainio et al., *Cell Mol Neurobiol* 14(5):439-457 (1994)) and at which polypeptides are not expected to bind. Thus, the present invention also provides for antisense oligonucleotides which are 10 complementary to allelic or homologous cDNAs and genomic DNAs corresponding to murine TLR9 nucleic acid containing SEQ ID NO:1 or SEQ ID NO:2.

In one set of embodiments, the antisense oligonucleotides of the invention may be composed of "natural" deoxyribonucleotides, ribonucleotides, or any combination thereof. That is, the 5' end of one native nucleotide and the 3' end of another native nucleotide may be 15 covalently linked, as in natural systems, via a phosphodiester internucleoside linkage. These oligonucleotides may be prepared by art-recognized methods which may be carried out manually or by an automated synthesizer. They also may be produced recombinantly by vectors.

In preferred embodiments, however, the antisense oligonucleotides of the invention 20 also may include "modified" oligonucleotides. That is, the oligonucleotides may be modified in a number of ways which do not prevent them from hybridizing to their target but which enhance their stability or targeting or which otherwise enhance their therapeutic effectiveness.

The term "modified oligonucleotide" as used herein describes an oligonucleotide in which (1) at least two of its nucleotides are covalently linked via a synthetic internucleoside 25 linkage (i.e., a linkage other than a phosphodiester linkage between the 5' end of one nucleotide and the 3' end of another nucleotide) and/or (2) a chemical group not normally associated with nucleic acids has been covalently attached to the oligonucleotide. Preferred synthetic internucleoside linkages are phosphorothioates, alkylphosphonates, phosphorodithioates, phosphate esters, alkylphosphonothioates, phosphoramidates, carbamates, carbonates, phosphate triesters, acetamidates, carboxymethyl esters and peptides. 30

The term "modified oligonucleotide" also encompasses oligonucleotides with a

covalently modified base and/or sugar. For example, modified oligonucleotides include oligonucleotides having backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified oligonucleotides may include a 2'-O-  
5 alkylated ribose group. In addition, modified oligonucleotides may include sugars such as arabinose instead of ribose. The present invention, thus, contemplates pharmaceutical preparations containing modified antisense molecules that are complementary to and hybridizable with, under physiological conditions, nucleic acids encoding murine TLR9 polypeptides, together with pharmaceutically acceptable carriers.

10 Agents which bind murine TLR9 also include binding peptides and other molecules which bind to the murine TLR9 polypeptide and complexes containing the murine TLR9 polypeptide. When the binding molecules are inhibitors, the molecules bind to and inhibit the activity of murine TLR9. When the binding molecules are activators, the molecules bind to and increase the activity of murine TLR9. To determine whether a murine TLR9 binding  
15 agent binds to murine TLR9 any known binding assay may be employed. For example, the binding agent may be immobilized on a surface and then contacted with a labeled murine TLR9 polypeptide. The amount of murine TLR9 which interacts with the murine TLR9 binding agent or the amount which does not bind to the murine TLR9 binding agent may then be quantitated to determine whether the murine TLR9 binding agent binds to murine TLR9.

20 The murine TLR9 binding agents include molecules of numerous size and type that bind selectively or preferentially to murine TLR9 polypeptides, and complexes of both murine TLR9 polypeptides and their binding partners. These molecules may be derived from a variety of sources. For example, murine TLR9 binding agents can be provided by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or  
25 as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

30 Phage display can be particularly effective in identifying binding peptides useful according to the invention. Briefly, one prepares a phage library (using, e.g., m13, fd, or lambda phage), displaying inserts from 4 to about 80 amino acid residues using conventional procedures. The inserts may represent, for example, a completely degenerate or biased array.

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One then can select phage-bearing inserts which bind to the murine TLR9 polypeptide. This process can be repeated through several cycles of reselection of phage that bind to the murine TLR9 polypeptide. Repeated rounds lead to enrichment of phage bearing particular sequences. DNA sequence analysis can be conducted to identify the sequences of the expressed polypeptides. The minimal linear portion of the sequence that binds to the murine TLR9 polypeptide can be determined. One can repeat the procedure using a biased library containing inserts containing part or all of the minimal linear portion plus one or more additional degenerate residues upstream or downstream thereof. Yeast two-hybrid screening methods also may be used to identify polypeptides that bind to the murine TLR9 polypeptides. Thus, the murine TLR9 polypeptides of the invention, or a fragment thereof, can be used to screen peptide libraries, including phage display libraries, to identify and select peptide binding partners of the murine TLR9 polypeptides of the invention. Such molecules can be used, as described, for screening assays, for purification protocols, for interfering directly with the functioning of murine TLR9 and for other purposes that will be apparent to those of ordinary skill in the art.

The invention also embraces agents which bind selectively to certain regulatory sequences associated with the murine TLR9 nucleic acid molecules described herein. The agents include polypeptides which bind to transcription and translation regulatory sequences of murine TLR9, and antisense nucleic acids, both of which are described in greater detail below. The agents can inhibit or increase murine TLR9 expression, as well as signaling activity (antagonists and agonists, respectively). Agents which bind selectively to regulatory sequences associated with the murine TLR9 nucleic acid molecules can be identified using methods familiar to those of skill in the art. For example, a promoter region including at least 100, 200, 300, 400, 500, or more nucleotides upstream (5') of the coding region of murine TLR9 can be identified by isolating, from appropriate genomic DNA, such nucleotide sequences using the sequences of SEQ ID NO:1 or SEQ ID NO:2 as primers or as probes, and then inserting the promoter region DNA into an appropriate expression vector so as to control the expression of TLR9 or some other reporter gene, introducing the TLR9 promoter vector into an appropriate host cell, and screening for TLR9 or reporter expression by those cells following their incubation in the presence and absence of various test agents. A reporter gene other than TLR9 can include, for example, an enzyme, a cytokine, a cell surface antigen,

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luciferase, chloramphenicol acetyl transferase (CAT), etc. An agent that inhibits expression of TLR9 or the reporter under the control of the TLR9 promoter is classified as a TLR9 expression inhibitor. Conversely, an agent that augments expression of TLR9 or reporter under the control of the TLR9 promoter is classified as a TLR9 expression enhancer. It was  
5 discovered according to the instant invention, for example, that the cytokine IL-4 inhibits the expression of TLR9. In this manner it is possible to identify agents that can be administered in conjunction with ISNA, for example by local administration, to enhance response to the ISNA. Such an enhancing effect might be desirable, for example, in the setting of immunization or vaccination. Conversely, it is possible to identify agents that can be  
10 administered in conjunction with a ISNA, for example by local administration, to inhibit response to the ISNA. Such an inhibiting response might be desirable, for example, in the setting of gene replacement therapy.

Therefore the invention generally provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions  
15 associated with TLR9 activity and the compounds and agents so identified. Generally, the screening methods involve assaying for compounds which inhibit or enhance signaling through murine TLR9. Such methods are adaptable to automated, high throughput screening of compounds. Examples of such high throughput screening methods are described in U.S. patents 6,103,479; 6,051,380; 6,051,373; 5,998,152; 5,876,946; 5,708,158; 5,443,791;  
20 5,429,921; and 5,143,854.

A variety of assays for pharmacological agents are provided, including labeled *in vitro* protein binding assays, signaling assays using detectable molecules, etc. For example, protein binding screens are used to rapidly examine the binding of candidate pharmacological agents to a murine TLR9. The candidate pharmacological agents can be derived from, for example,  
25 combinatorial peptide or nucleic acid libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of signaling involves contacting a cell having a murine TLR9 with a candidate pharmacological agent under conditions whereby the induction of a detectable molecule can occur. Specific conditions are well known in the art and are described, for example, in Häcker H et al., *J Exp Med* 192:595-600 (2000), and  
30 references cited therein. A reduced degree of induction of the detectable molecule in the presence of the candidate pharmacological agent indicates that the candidate pharmacological

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agent reduces the signaling activity of murine TLR9. An increased degree of induction of the detectable molecule in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent increases the signaling activity of murine TLR9.

Murine TLR9 used in the methods of the invention can be added to an assay mixture  
5 as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a murine TLR9 polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the murine TLR9 as a polypeptide or as a nucleic acid (e.g., a cell transfected with an expression vector containing a murine TLR9). In the assays described herein, the  
10 murine TLR9 polypeptide can be produced recombinantly, isolated from biological extracts, or synthesized *in vitro*. Murine TLR9 polypeptides encompass chimeric proteins comprising a fusion of a murine TLR9 polypeptide with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, enhancing signaling capability, facilitating detection, or enhancing stability of the murine TLR9 polypeptide under assay conditions. A  
15 polypeptide fused to a murine TLR9 polypeptide or fragment thereof may also provide means of readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

The assay mixture also comprises a candidate pharmacological agent. Typically, a plurality of assay mixtures are run in parallel with different agent concentrations to obtain a  
20 different response to the various concentrations. Typically, one of these concentrations serves as a negative control, i.e., at zero concentration of agent or at a concentration of agent below the limits of assay detection. Candidate pharmaceutical agents encompass numerous chemical classes, although typically they are organic compounds. Preferably, the candidate pharmacological agents are small organic compounds, i.e., those having a molecular weight  
25 of more than 50 yet less than about 2500. Polymeric candidate agents can have higher molecular weights, e.g., oligonucleotides in the range of about 2500 to about 12,500.

Candidate agents comprise functional chemical groups necessary for structural interactions with polypeptides, and may include at least an amine, carbonyl, hydroxyl or carboxyl group, preferably at least two of the functional chemical groups and more preferably at least three of  
30 the functional chemical groups. The candidate agents can comprise cyclic carbon or heterocyclic structure and/or aromatic or polyaromatic structures substituted with one or more

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of the above-identified functional groups. Candidate agents also can be biomolecules such as nucleic acids, peptides, saccharides, fatty acids, sterols, isoprenoids, purines, pyrimidines, derivatives or structural analogs of the above, or combinations thereof and the like. Where the agent is a nucleic acid, the agent typically is a DNA or RNA molecule, although modified 5 nucleic acids having non-natural bonds or subunits are also contemplated.

Candidate agents are obtained from a wide variety of sources, including libraries of natural, synthetic, or semisynthetic compounds, or any combination thereof. For example, numerous means are available for random and directed synthesis of a wide variety of organic compounds and biomolecules, including expression of randomized oligonucleotides, 10 synthetic organic combinatorial libraries, phage display libraries of random peptides, and the like. Alternatively, libraries of natural compounds in the form of bacterial, fungal, plant and animal extracts are available or readily produced. Additionally, natural and synthetically produced libraries and compounds can be readily modified through conventional chemical, physical, and biochemical means. Further, known pharmacological agents may be subjected 15 to directed or random chemical modifications such as acylation, alkylation, esterification, amidification, etc., to produce structural analogs of the agents.

Therefore, a source of candidate agents are libraries of molecules based on known TLR9 ligands, e.g., CpG oligonucleotides shown herein to interact with TLR9, in which the structure of the ligand is changed at one or more positions of the molecule to contain more or 20 fewer chemical moieties or different chemical moieties. The structural changes made to the molecules in creating the libraries of analog inhibitors can be directed, random, or a combination of both directed and random substitutions and/or additions. One of ordinary skill in the art in the preparation of combinatorial libraries can readily prepare such libraries based on existing TLR9 ligands.

25 A variety of other reagents also can be included in the mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease 30 inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby,

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but for the presence of the candidate pharmacological agent, the murine TLR9 mediates TLR/IL-1R signaling. For determining the binding of a candidate pharmaceutical agent to a murine TLR9, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of  
5 the assay may be readily determined. Such experimentation merely involves optimization of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the level of signaling or the level of specific binding between the  
10 murine TLR9 polypeptide and the candidate pharmaceutical agent is detected by any convenient method available to the user. For cell-free binding type assays, a separation step is often used to separate bound from unbound components. The separation step may be accomplished in a variety of ways. For example, separation can be accomplished in solution, or, conveniently, at least one of the components is immobilized on a solid substrate, from  
15 which the unbound components may be easily separated. The solid substrate can be made of a wide variety of materials and in a wide variety of shapes, e.g., microtiter plate, microbead, dipstick, resin particle, etc. The substrate preferably is chosen to maximize signal-to-noise ratios, primarily to minimize background binding, as well as for ease of separation and cost.

Separation may be effected for example, by removing a bead or dipstick from a  
20 reservoir, emptying or diluting a reservoir such as a microtiter plate well, rinsing a bead, particle, chromatographic column or filter with a wash solution or solvent. The separation step preferably includes multiple rinses or washes. For example, when the solid substrate is a microtiter plate, the wells may be washed several times with a washing solution, which typically includes those components of the incubation mixture that do not participate in  
25 specific bindings such as salts, buffer, detergent, non-specific protein, etc. Where the solid substrate is a magnetic bead, the beads may be washed one or more times with a washing solution and isolated using a magnet.

Detection may be effected in any convenient way for cell-based assays such as measurement of an induced polypeptide within, on the surface of, or secreted by the cell.  
30 Examples of detection methods useful in such cell-based assays include fluorescence-activated cell sorting (FACS) analysis, bioluminescence, fluorescence, enzyme-linked

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immunosorbent assay (ELISA), reverse transcriptase-polymerase chain reaction (RT-PCR), and the like.

A variety of methods may be used to detect the label, depending on the nature of the label and other assay components. For example, the label may be detected while bound to the solid substrate or subsequent to separation from the solid substrate. Labels may be directly detected through optical or electron density, radioactive emissions, nonradiative energy transfers, etc., or indirectly detected with antibody conjugates, streptavidin-biotin conjugates, etc. Methods for detecting the labels are well known in the art.

The murine TLR9 binding agent may also be an antibody or a functionally active antibody fragment. Antibodies are well known to those of ordinary skill in the science of immunology. As used herein, the term "antibody" means not only intact antibody molecules but also fragments of antibody molecules retaining specific target binding ability. Such fragments are also well known in the art and are regularly employed both *in vitro* and *in vivo*. In particular, as used herein, the term "antibody" means not only intact immunoglobulin molecules but also the well-known active fragments  $F(ab')_2$  and Fab.  $F(ab')_2$  and Fab fragments which lack the Fc fragment of intact antibody clear more rapidly from the circulation and may have less non-specific tissue binding than an intact antibody (Wahl RL et al., *J Nucl Med* 24:316-325 (1983)).

Monoclonal antibodies may be made by any of the methods known in the art utilizing murine TLR9, or a fragment thereof, as an immunogen. Alternatively the antibody may be a polyclonal antibody specific for murine TLR9 which inhibits murine TLR9 activity. The preparation and use of polyclonal antibodies are also known to one of ordinary skill in the art.

Significantly, as is well known in the art, only a small portion of an antibody molecule, the paratope, is involved in the binding of the antibody to its epitope (see, in general, Clark, W.R. (1986) *The Experimental Foundations of Modern Immunology*, Wiley & Sons, Inc., New York; Roitt, I. (1991) *Essential Immunology*, 7th Ed., Blackwell Scientific Publications, Oxford). The pFc' and Fc regions, for example, are effectors of the complement cascade but are not involved in antigen binding. An antibody from which the pFc' region has been enzymatically cleaved, or which has been produced without the pFc' region, designated an  $F(ab')_2$  fragment, retains both of the antigen binding sites of an intact antibody. Similarly, an antibody from which the Fc region has been enzymatically cleaved, or which has been

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produced without the Fc region, designated an Fab fragment, retains one of the antigen binding sites of an intact antibody molecule. Proceeding further, Fab fragments consist of a covalently bound antibody light chain and a portion of the antibody heavy chain denoted Fd. The Fd fragments are the major determinant of antibody specificity (a single Fd fragment may 5 be associated with up to ten different light chains without altering antibody specificity) and Fd fragments retain epitope-binding ability in isolation.

Within the antigen-binding portion of an antibody, as is well-known in the art, there are complementarity determining regions (CDRs), which directly interact with the epitope of the antigen, and framework regions (FRs), which maintain the tertiary structure of the 10 paratope (see, in general, Clark, 1986; Roitt, 1991). In both the heavy chain Fd fragment and the light chain of IgG immunoglobulins, there are four framework regions (FR1 through FR4) separated respectively by three complementarity determining regions (CDR1 through CDR3). The CDRs, and in particular the CDR3 regions, and more particularly the heavy chain CDR3, are largely responsible for antibody specificity.

15 The sequences of the antigen-binding Fab' portion of the anti-murine TLR9 monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing methods that are routine in the art. It is well established that non-CDR regions of a mammalian antibody may be replaced with corresponding regions of non-specific 20 or hetero-specific antibodies while retaining the epitope specificity of the original antibody. This technique is useful for the development and use of "humanized" antibodies in which non-human CDRs are covalently joined to human FR and/or Fc/pFc' regions to produce a functional antibody. Techniques to humanize antibodies are particularly useful when non-human animal (e.g., murine) antibodies which inhibit murine TLR9 activity are identified. 25 These non-human animal antibodies can be humanized for use in the treatment of a human subject in the methods according to the invention. Examples of methods for humanizing a murine antibody are provided in U.S. patents 4,816,567, 5,225,539, 5,585,089, 5,693,762 and 5,859,205. Other antibodies, including fragments of intact antibodies with antigen-binding ability, are often referred to as "chimeric" antibodies.

30 Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')<sub>2</sub> and Fab fragments of an anti-murine TLR9 monoclonal antibody;

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chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-murine TLR9 antibody have been replaced by homologous human or non-human sequences; chimeric F(ab')<sub>2</sub> fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-murine TLR9 antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences.

According to the invention murine TLR9 inhibitors also include "dominant negative" polypeptides derived from SEQ ID NO:3. A dominant negative polypeptide is an inactive variant of a polypeptide, which, by interacting with the cellular machinery, displaces an active polypeptide from its interaction with the cellular machinery or competes with the active polypeptide, thereby reducing the effect of the active polypeptide. For example, a dominant negative receptor which binds a ligand but does not transmit a signal in response to binding of the ligand can reduce the biological effect of expression of the receptor. As shown in the Examples below, TLR9 polypeptides which incorporate the substitution of histidine for proline at aa 915 (P915H mutation) are functionally inactive and are dominant negative with respect to the native TLR9 polypeptide.

The end result of the expression of a dominant negative murine TLR9 polypeptide of the invention in a cell is a reduction in TLR9 activity such as signaling through the TIR pathway. One of ordinary skill in the art can assess the potential for a dominant negative variant of a murine TLR9 polypeptide and, using standard mutagenesis techniques, create one or more dominant negative variant polypeptides. For example, given the teachings contained herein of a murine TLR9 polypeptide, one of ordinary skill in the art can modify the sequence of the murine TLR9 polypeptide by site-specific mutagenesis, scanning mutagenesis, partial gene deletion or truncation, and the like. See, e.g., U.S. Patent No. 5,580,723 and *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989. The skilled artisan then can test the population of mutagenized polypeptides for diminution in murine TLR9 activity and/or for retention of such an activity. Other similar methods for creating and testing dominant negative variants of a murine TLR9 polypeptide will be apparent to one of ordinary skill in the art.

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Each of the compositions according to this aspect of the invention is useful for a variety of therapeutic and non-therapeutic purposes. For example, the murine TLR9 nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. A suitable oligonucleotide or set of oligonucleotides, which is capable of hybridizing under stringent hybridization conditions to the desired sequence, a variant or fragment thereof, or an anti-sense complement of such an oligonucleotide or set of oligonucleotides, can be synthesized by means well known in the art (see, for example, *Synthesis and Application of DNA and RNA*, S.A. Narang, ed., 1987, Academic Press, San Diego, CA) and employed as a probe to identify and isolate the desired sequence, variant or fragment thereof by techniques known in the art. Techniques of nucleic acid hybridization and clone identification are disclosed by Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

To facilitate the detection of a desired nucleic acid sequence, or variant or fragment thereof, whether for cloning purposes or for the mere detection of the presence of the sequence, the above-described probes may be labeled with a detectable group. Such a detectable group may be any material having a detectable physical or chemical property. Such materials have been well developed in the field of nucleic acid hybridization and, in general, many labels useful in such methods can be applied to the present invention. Particularly useful are radioactive labels. Any radioactive label may be employed which provides for an adequate signal and has a sufficient half-life. If single stranded, the oligonucleotide may be radioactively labeled using kinase reactions. Alternatively, oligonucleotides are also useful as nucleic acid hybridization probes when labeled with a non-radioactive marker such as biotin, an enzyme or a fluorescent group. See, for example, Leary JJ et al., *Proc Natl Acad Sci USA* 80:4045 (1983); Renz M et al., *Nucleic Acids Res* 12:3435 (1984); and Renz M, *EMBO J* 6:817 (1983).

Additionally, complements of the murine TLR9 nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a murine TLR9 "knockout" phenotype. The administration of antisense RNA probes to block gene expression is discussed in Lichtenstein C, *Nature* 333:801-802 (1988).

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Alternatively, the murine TLR9 nucleic acid of the invention can be used to prepare a non-human transgenic animal. A "transgenic animal" is an animal having cells that contain DNA which has been artificially inserted into a cell, which DNA becomes part of the genome of the animal which develops from that cell. Preferred transgenic animals are primates, mice, rats, cows, pigs, horses, goats, sheep, dogs and cats. Animals suitable for transgenic experiments can be obtained from standard commercial sources such as Charles River (Wilmington, MA), Taconic (Germantown, NY), Harlan (Indianapolis, IN), etc. Transgenic animals having a particular property associated with a particular disease can be used to study the effects of a variety of drugs and treatment methods on the disease, and thus serve as genetic models for the study of a number of human diseases. The invention, therefore, contemplates the use of murine TLR9 knockout and transgenic animals as models for the study of disorders involving TLR9-mediated signaling. A variety of methods known to one of ordinary skill in the art are available for the production of transgenic animals associated with this invention.

Inactivation or replacement of the endogenous TLR9 gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-human mammals having a TLR9<sup>-/-</sup> knockout phenotype may be made transgenic for the murine TLR9 and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the murine TLR9. In this manner, such therapeutic drugs can be identified.

Additionally, a normal or mutant version of murine TLR9 can be inserted into the germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of murine TLR9. These animals are useful in studies to define the role and function of murine TLR9 in cells.

Generally, doses of active compounds would be from about 0.01 mg/kg per day to 1000 mg/kg per day. It is expected that doses ranging from 50-500 mg/kg will be suitable and in one or several administrations per day. Lower doses will result from other forms of administration, such as intravenous administration. In the event that a response in a subject is insufficient at the initial doses applied, higher doses (or effectively higher doses by a different, more localized delivery route) may be employed to the extent that patient tolerance permits. Multiple doses per day are contemplated to achieve appropriate systemic levels of

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compound, although fewer doses typically will be given when compounds are prepared as slow release or sustained release medications.

The antagonists, agonists, nucleic acids, and polypeptides of murine TLR9 useful according to the invention may be combined, optionally, with a pharmaceutically acceptable carrier. Thus the invention also provides pharmaceutical compositions and a method for preparing the pharmaceutical compositions which contain compositions of this aspect of the invention. The pharmaceutical compositions include any one or combination of the antagonists, agonists, nucleic acids and polypeptides of murine TLR9 useful according to the invention and, optionally, a pharmaceutically acceptable carrier. Each pharmaceutical composition is prepared by selecting an antagonist, agonist, nucleic acid or polypeptide of murine TLR9 useful according to the invention, as well as any combination thereof, and, optionally, combining it with a pharmaceutically acceptable carrier.

The term "pharmaceutically acceptable carrier" as used herein means one or more compatible solid or liquid fillers, diluents or encapsulating substances which are suitable for administration into a human. The term "carrier" denotes an organic or inorganic ingredient, natural or synthetic, with which the active ingredient is combined to facilitate the application. The components of the pharmaceutical compositions also are capable of being co-mingled with the molecules of the present invention, and with each other, in a manner such that there is no interaction which would substantially impair the desired pharmaceutical efficacy.

The pharmaceutical compositions may contain suitable buffering agents, including, without limitation: acetic acid in a salt; citric acid in a salt; and phosphoric acid in a salt.

The pharmaceutical compositions also may contain, optionally, suitable preservatives, such as benzalkonium chloride, chlorobutanol, parabens, and thimerosal.

When administered, the pharmaceutical preparations of the invention are applied in pharmaceutically acceptable amounts and in pharmaceutically acceptable compositions. Such preparations may routinely contain salts, buffering agents, preservatives, compatible carriers, and optionally other therapeutic agents. When used in medicine, the salts should be pharmaceutically acceptable, but non-pharmaceutically acceptable salts may conveniently be used to prepare pharmaceutically acceptable salts thereof and are not excluded from the scope of the invention. Such pharmacologically and pharmaceutically acceptable salts include, but are not limited to, those prepared from the following acids: hydrochloric, hydrobromic,

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sulfuric, nitric, phosphoric, maleic, acetic, salicylic, citric, formic, malonic, succinic, and the like. Also, pharmaceutically acceptable salts can be prepared as alkaline metal or alkaline earth salts, such as sodium, potassium or calcium salts.

A variety of administration routes are available. The particular mode selected will 5 depend, of course, upon the particular compound selected, the severity of the condition being treated, and the dosage required for therapeutic efficacy. The methods of the invention, generally speaking, may be practiced using any mode of administration that is medically acceptable, meaning any mode that produces effective levels of the active compounds without causing clinically unacceptable adverse effects. Such modes of administration include oral, 10 rectal, topical, nasal, intradermal, or parenteral routes. The term "parenteral" includes, without limitation, subcutaneous, transdermal, intravenous, intra-arterial, intrathecal, intramuscular, intraperitoneal, mucosal (apart from gastrointestinal mucosa), pulmonary, intralesional, and infusion.

The pharmaceutical compositions may conveniently be presented in unit dosage form 15 and may be prepared by any of the methods well-known in the art of pharmacy. All methods include the step of bringing the active agent into association with a carrier which constitutes one or more accessory ingredients. In general, the compositions are prepared by uniformly and intimately bringing the active compound into association with a liquid carrier, a finely divided solid carrier, or both, and then, if necessary, shaping the product.

20 Compositions suitable for oral administration may be presented as discrete units, such as capsules, tablets, lozenges, each containing a predetermined amount of the active compound. Other compositions include suspensions in aqueous liquids or non-aqueous liquids such as a syrup, elixir or an emulsion.

Compositions suitable for parenteral administration conveniently comprise a sterile 25 aqueous preparation of the antagonists, agonists, nucleic acids, or polypeptides of murine TLR9, which is preferably isotonic with the blood of the recipient. This aqueous preparation may be formulated according to known methods using suitable dispersing or wetting agents and suspending agents. The sterile injectable preparation also may be a sterile injectable solution or suspension in a non-toxic parenterally acceptable diluent or solvent, for example, 30 as a solution in 1,3-butane diol. Among the acceptable vehicles and solvents that may be employed are water, Ringer's solution, and isotonic sodium chloride solution. In addition,

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sterile, fixed oils are conventionally employed as a solvent or suspending medium. For this purpose any bland fixed oil may be employed including synthetic mono- or di-glycerides. In addition, fatty acids such as oleic acid may be used in the preparation of injectables. Carrier formulation suitable for oral, subcutaneous, intravenous, intrathecal, intramuscular, etc.

- 5    administrations can be found in Remington's Pharmaceutical Sciences, Mack Publishing Co.,  
Easton, PA.

Other delivery systems can include time-release, delayed release or sustained release delivery systems such as the biological/chemical vectors is discussed above. Such systems can avoid repeated administrations of the active compound, increasing convenience to the  
10    subject and the physician. Many types of release delivery systems are available and known to those of ordinary skill in the art. Use of a long-term sustained release implant may be desirable. Long-term release, are used herein, means that the implant is constructed and arranged to delivery therapeutic levels of the active ingredient for at least 30 days, and  
15    preferably 60 days. Long-term sustained release implants are well-known to those of ordinary skill in the art and include some of the release systems described above.

In another aspect the invention involves the identification of cDNAs encoding mouse TLR7 and mouse TLR8, referred to herein as murine TLR7 and murine TLR8 and, equivalently, mTLR7 and mTLR8, respectively. The nucleotide sequence of the cDNA for murine TLR7 is presented as SEQ ID NO:173, the coding region of the cDNA for murine  
20    TLR7 is presented as SEQ ID NO:174, and the amino acid sequence of the murine TLR7 is presented as SEQ ID NO:175. The closely related human TLR7 (equivalently, hTLR7) was previously deposited in GenBank under accession numbers AF245702 and AF240467. The nucleotide sequence of the cDNA for murine TLR7 presented as SEQ ID NO:173 is 3357  
nucleotides long and includes the ORF spanning bases 117-3266, presented as SEQ ID  
25    NO:174, which spans 3150 nucleotides (excluding the stop codon). The amino acid sequence of the murine TLR7 presented as SEQ ID NO:175 is 1050 amino acids long.

The nucleotide sequence of the cDNA for murine TLR8 is presented as SEQ ID NO:190, the coding region of the cDNA for murine TLR8 is presented as SEQ ID NO:191, and the amino acid sequence of the murine TLR8 is presented as SEQ ID NO:192. The  
30    closely related human TLR8 (equivalently, hTLR8) was previously deposited in GenBank under accession numbers AF245703 and AF246971.

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Like both human and murine TLR9, human TLR7 and human TLR8 each contains one CXXC motif and one MBD motif. The hTLR7 CXXC motif contains amino acids 258-273, and the hTLR8 CXXC motif contains amino acids 255-270.

5

	CXXC motif:	GNCXXCXXXXXXCXXC	SEQ ID NO:196
	hTLR9:	GNCRRCDHAPNPCMEC	SEQ ID NO:197
	mTLR9:	GNCRRCDHAPNPCMIC	SEQ ID NO:198
	hTLR7:	GNCPRCYNAPFPCAPC	SEQ ID NO:199
10	mTLR7:	GNCPRCYNVPYPCTPC	SEQ ID NO:200
	hTLR8:	GNCPRCFNAPFPCVPC	SEQ ID NO:201
	mTLR8:	GNCPRCYNAPFPCTPC	SEQ ID NO:202

Also like human and murine TLR9, human TLR7 and TLR8 also have a single MBD motif. The the hTLR7 MBD motif spans amino acids 545-575, and the hTLR8 MBD motif amino acids spans 533-563.

#### MBD motif

	MBD-1	R-XXXXXXXX-R-X-D-X-Y-XXXXXXXXXX-R-S-XXXXXX-Y	SEQ ID NO:125
20	hTLR9	Q-XXXXXXXX-K-X-D-X-Y-XXXXXXXXXX-R-L-XXXXXX-Y	SEQ ID NO:126
	mTLR9	Q-XXXXXXXX-K-X-D-X-Y-XXXXXXXXXX-Q-L-XXXXXX-Y	SEQ ID NO:127
	hTLR7	R-XXXXXXXX-R-X-D-X-L-XXXXXXXXXX-K-L-XXXXXX-S	SEQ ID NO:203
	mTLR7	R-XXXXXXXX-R-X-D-X-L-XXXXXXXXXX-S-L-XXXXXX-S	SEQ ID NO:204
	hTLR8	K-XXXXXXXX-R-X-D-X-D-XXXXXXXXXX-D-L-XXXXXX-Y	SEQ ID NO:205
25	mTLR8	K-XXXXXXXX-R-X-D-X-D-XXXXXXXXXX-D-L-XXXXXX-H	SEQ ID NO:206
	hTLR7	R-YLDFSNN-R-L-D-L-L-HSTAFEELH-K-L-EVLDIS-S	SEQ ID NO:212
	mTLR7	R-YLDFSNN-R-L-D-L-L-YSTAFEELQ-S-L-EVLDLS-S	SEQ ID NO:213
30	hTLR8	K-YLDLTNN-R-L-D-F-D-NASALTELS-D-L-EVLDLS-Y	SEQ ID NO:214
	mTLR8	K-YLDLTNN-R-L-D-F-D-DNNAFSDLH-D-L-EVLDLS-H	SEQ ID NO:215

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The core D-X-Y in the MBD motif is involved in CpG binding of the MBD-1 protein and is conserved in TLR9 but only partially conserved in TLR8 and TLR7 (Y to D or L). The other mismatches are highly or moderately conserved; example R to K, Q, or D. These  
5 changes could explain MBD-1 as a methyl-CpG binder and TLR9 as a binder for CpG-DNA. The modification in the core sequence (D-X-Y) in hTLR7 (D-X-L) and TLR8 (D-X-D) is likely a structural basis for the recognition of different nucleic acid motifs. Combined with the presence of a CXXC domain TLR7 and TLR8 appear certainly to be nucleic acid binding receptors relevant to the innate immune system and thus clinical value.

10 The invention involves in one aspect murine TLR7 and murine TLR8 nucleic acids and polypeptides, as well as therapeutics relating thereto. The invention also embraces isolated functionally equivalent variants, useful analogs and fragments of the foregoing murine TLR7 and murine TLR8 nucleic acids and polypeptides; complements of the foregoing murine TLR7 and murine TLR8 nucleic acids; and molecules which selectively  
15 bind the foregoing murine TLR7 and murine TLR8 nucleic acids and polypeptides.

The murine TLR7 and murine TLR8 nucleic acids and polypeptides of the invention are isolated. The term "isolated," with respect to murine TLR7 and murine TLR8 nucleic acids and polypeptides, has the same meaning as used elsewhere herein.

20 As used herein a murine TLR7 nucleic acid refers to an isolated nucleic acid molecule which codes for a murine TLR7 polypeptide. Such nucleic acid molecules code for murine TLR7 polypeptides which include the sequence of SEQ ID NO:175 and fragments thereof. The nucleic acid molecules include the nucleotide sequences of SEQ ID NO:173, SEQ ID NO:174, and nucleotide sequences which differ from the sequences of SEQ ID NO:173 and SEQ ID NO:174 in codon sequence due to the degeneracy of the genetic code.

25 Also as used herein a murine TLR8 nucleic acid refers to an isolated nucleic acid molecule which codes for a murine TLR8 polypeptide. Such nucleic acid molecules code for murine TLR8 polypeptides which include the sequences of SEQ ID NO:193, and fragments thereof. The nucleic acid molecules include the nucleotide sequences of SEQ ID NO:190, SEQ ID NO:191, and nucleotide sequences which differ from the sequences of SEQ ID  
30 NO:190 and SEQ ID NO:191 in codon sequence due to the degeneracy of the genetic code.

The murine TLR7 and murine TLR8 nucleic acids of the invention also include alleles

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as well as fragments of the foregoing nucleic acids. Such fragments can be used, for example, as probes in hybridization assays and as primers in a polymerase chain reaction. Preferred murine TLR7 nucleic acids include the nucleic acid sequence of SEQ ID NO:173 and SEQ ID NO:174. Preferred murine TLR8 nucleic acids include the nucleic acid sequence 5 of SEQ ID NO:190 and SEQ ID NO:191. Complements of the foregoing nucleic acids also are embraced by the invention.

As used herein a murine TLR7 nucleic acid or murine TLR7 polypeptide also embraces homologues and alleles of murine TLR7. Likewise, as used herein a murine TLR8 nucleic acid or murine TLR8 polypeptide also embraces homologues and alleles of murine 10 TLR8. Homologues and alleles of murine TLR7 and murine TLR8 comply with the degrees of nucleotide and amino acid identity as previously set forth herein in reference to homologues and alleles of murine TLR9.

Alleles of the murine TLR7 and murine TLR8 nucleic acids of the invention can be identified by conventional techniques. For example, alleles of murine TLR7 can be isolated 15 by hybridizing a probe which includes at least a fragment of SEQ ID NO:173 or SEQ ID NO:174 under stringent conditions with a cDNA library and selecting positive clones. Thus, an aspect of the invention is those nucleic acid sequences which code for murine TLR7 polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:173 or SEQ ID NO:174 under stringent conditions. Likewise, an aspect of the invention is those 20 nucleic acid sequences which code for murine TLR8 polypeptides and which hybridize to a nucleic acid molecule consisting of SEQ ID NO:190 or SEQ ID NO:191 under stringent conditions. Stringent conditions in this context has the same meaning as described elsewhere herein, including the use of a suitable hybridization buffer and a temperature of about 65°C.

In screening for murine TLR7 or murine TLR8 nucleic acids, a Southern blot may be 25 performed using the stringent conditions previously described herein, together with a radioactive probe. After washing the membrane to which the DNA is finally transferred, the membrane can be placed against X-ray film to detect the radioactive signal. Corresponding non-radioactive methods are also well known in the art and can be used to similar effect.

The murine TLR7 and murine TLR8 nucleic acids of the invention also include 30 degenerate nucleic acids which include alternative codons to those present in the native materials, as previously described herein.

The invention also provides modified nucleic acid molecules which include additions, substitutions and deletions of one or more nucleotides. The modified nucleic acid molecules according to this aspect of the invention exclude fully native human TLR7 (SEQ ID NO:168,

5 SEQ ID NO:169, GenBank Accession No. AF245702, and GenBank Accession No. AF240467) and fully native human TLR8 nucleic acid molecules (SEQ ID NO:182, SEQ ID NO:183, GenBank Accession No. AF245703, and GenBank Accession No. AF246971). In preferred embodiments, these modified nucleic acid molecules and/or the polypeptides they encode retain at least one activity or function of the unmodified nucleic acid molecule and/or 10 the polypeptides, such as signaling activity, etc. In certain embodiments, the modified nucleic acid molecules encode modified polypeptides, preferably polypeptides having conservative amino acid substitutions as are described elsewhere herein. The modified nucleic acid molecules are structurally related to the unmodified nucleic acid molecules and in preferred 15 embodiments are sufficiently structurally related to the unmodified nucleic acid molecules so that the modified and unmodified nucleic acid molecules hybridize under stringent conditions known to one of skill in the art.

The invention also provides isolated fragments of nucleotide sequences for murine TLR7 (SEQ ID NO:173 and SEQ ID NO:174) and for murine TLR8 (SEQ ID NO:190 and SEQ ID NO:191). The fragments can be used as probes in Southern blot assays to identify 20 such nucleic acids, or can be used in amplification assays such as those employing PCR. Smaller fragments are those comprising 12, 13, 14, 15, 16, 17, 18, 20, 22, 25, 30, 40, 50, or 75 nucleotides, and every integer therebetween, and are useful, e.g., as primers for nucleic acid amplification procedures. As known to those skilled in the art, larger probes such as 25 200, 250, 300, 400 or more nucleotides are preferred for certain uses such as Southern blots, while smaller fragments will be preferred for uses such as PCR. Fragments also can be used to produce fusion proteins for generating antibodies or determining binding of the polypeptide fragments. Likewise, fragments can be employed to produce non-fused fragments of the murine TLR7 and murine TLR8 polypeptides, useful, for example, in the preparation of 30 antibodies, in immunoassays, and the like. The foregoing nucleic acid fragments further can be used as antisense molecules to inhibit the expression of murine TLR7 and murine TLR8 nucleic acids and polypeptides.

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The invention also includes functionally equivalent variants of the murine TLR7 and murine TLR8, which include variant nucleic acids and polypeptides which retain one or more of the functional properties of the murine TLR7 and murine TLR8. Preferably such variants include the murine-specific N-terminal domain.

5 Functionally equivalent variants also include a murine TLR7 or murine TLR8 which has had a portion (e.g., of the N-terminus) removed or replaced by a similar domain from another TLR (e.g., a "domain-swapping" variant). Examples of such domain-swapping variants include those involving swapping a TLR7 domain from another species and swapping a TLR domain from another TLR.

10 Other functionally equivalent variants will be known to one of ordinary skill in the art, as will be methods for preparing such variants. The activity of a functionally equivalent variant can be determined using the methods provided herein, and in references that have described assays using other TLRs and TLRs of other species. Such variants are useful, *inter alia*, for evaluating bioavailability of drugs, in assays for identification of compounds which bind to and/or regulate the signaling function of the murine TLR7 and murine TLR8, and for determining the portions of the murine TLR7 and murine TLR8 which are required for 15 signaling activity.

20 Variants which are non-functional also can be prepared as described above. Such variants are useful, for example, as negative controls in experiments testing TLR7 and TLR8 signaling activity. Examples of non-functional variants include those incorporating a truncation or mutation of amino acids deemed critical to ligand binding or signaling activity.

25 In certain embodiments a murine TLR7 or murine TLR8 nucleic acid is operably linked to a gene expression sequence which can direct the expression of the murine TLR7 or murine TLR8 nucleic acid within a eukaryotic or prokaryotic cell. The terms "gene expression sequence" and "operably linked" are as previously described herein.

30 The murine TLR7 and murine TLR8 nucleic acid molecules and the murine TLR7 and murine TLR8 polypeptides of the invention can be delivered to a eukaryotic or prokaryotic cell alone or in association with a vector. As applied to murine TLR7 and murine TLR8 nucleic acid molecules, a "vector" is any vehicle capable of facilitating: (1) delivery of a murine TLR7 or murine TLR8 nucleic acid or polypeptide to a target cell, (2) uptake of a murine TLR7 or murine TLR8 nucleic acid or polypeptide by a target cell, or (3) expression

of a murine TLR7 or murine TLR8 nucleic acid molecule or polypeptide in a target cell.

In addition to the biological vectors, chemical/physical vectors may be used to deliver a murine TLR7 or murine TLR8 nucleic acid or polypeptide to a target cell and facilitate uptake thereby. As used herein with respect to a murine TLR7 or murine TLR8 nucleic acid or polypeptide, a "chemical/physical vector" refers to a natural or synthetic molecule, other than those derived from bacteriological or viral sources, capable of delivering the isolated murine TLR7 or murine TLR8 nucleic acid or polypeptide to a cell.

Other exemplary compositions that can be used to facilitate uptake by a target cell of the murine TLR7 or murine TLR8 nucleic acids include calcium phosphate and other chemical mediators of intracellular transport, microinjection compositions, electroporation and homologous recombination compositions (e.g., for integrating a murine TLR7 or murine TLR8 nucleic acid into a preselected location within a target cell chromosome).

It will also be recognized that the invention embraces the use of the murine TLR7 and murine TLR8 cDNA sequences in expression vectors to transfect host cells and cell lines, be these prokaryotic (e.g., *E. coli*), or eukaryotic (e.g., 293 fibroblast cells (ATCC, CRL-1573), MonoMac-6, THP-1, U927, CHO cells, COS cells, yeast expression systems and recombinant baculovirus expression in insect cells). Especially useful are mammalian cells such as human, pig, goat, primate, rodent, guinea pig, etc. They may be of a wide variety of tissue types, and include primary cells and cell lines. The expression vectors require that the pertinent sequence, i.e., those nucleic acids described *supra*, be operably linked to a promoter.

The invention also provides isolated murine TLR7 and isolated murine TLR8 polypeptides which include the amino acid sequences of SEQ ID NO:175, SEQ ID NO:192, and fragments thereof, encoded by the murine TLR7 and murine TLR8 nucleic acids described above. Murine TLR7 and murine TLR8 polypeptides also embrace alleles, functionally equivalent variants and analogs (those non-allelic polypeptides which vary in amino acid sequence from the disclosed murine TLR7 and murine TLR8 polypeptides by 1, 2, 3, 4, 5, or more amino acids) provided that such polypeptides retain murine TLR7 or murine TLR8 activity. Non-functional variants also are embraced by the invention; these are useful as antagonists of TLR7 and TLR8 signaling function, as negative controls in assays, and the like. Such alleles, variants, analogs and fragments are useful, for example, alone or as fusion proteins for a variety of purposes including as a component of assays.

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The invention also embraces variants of the murine TLR7 and murine TLR8 polypeptides described above. Modifications which create a murine TLR7 variant or murine TLR8 variant can be made to a murine TLR7 or murine TLR8 polypeptide for a variety of reasons, including 1) to reduce or eliminate an activity of a murine TLR7 or murine TLR8 polypeptide, such as signaling; 2) to enhance a property of a murine TLR7 or murine TLR8 polypeptide, such as signaling, binding affinity for nucleic acid ligand or other ligand molecule, protein stability in an expression system, or the stability of protein-protein binding; 3) to provide a novel activity or property to a murine TLR7 or murine TLR8 polypeptide, such as addition of an antigenic epitope or addition of a detectable moiety, e.g., luciferase, FLAG peptide, GFP; 4) to establish that an amino acid substitution does or does not affect molecular signaling activity; or 5) reduce immunogenicity. Modifications to a murine TLR7 or murine TLR8 polypeptide are typically made to the nucleic acid which encodes the murine TLR7 or murine TLR8 polypeptide, and can include deletions, point mutations, truncations, amino acid substitutions and additions of amino acids or non-amino acid moieties.

Alternatively, modifications can be made directly to the polypeptide, such as by cleavage, addition of a linker molecule, addition of a detectable moiety (for example, biotin, fluorophore, radioisotope, enzyme, or peptide), addition of a fatty acid, and the like.

Modifications also embrace fusion proteins comprising all or part of the murine TLR7 or murine TLR8 amino acid sequence.

Variants include murine TLR7 and murine TLR8 polypeptides which are modified specifically to alter a feature of each polypeptide unrelated to its physiological activity. For example, cysteine residues can be substituted or deleted to prevent unwanted disulfide linkages. Similarly, certain amino acids can be changed to enhance expression of a murine TLR7 or murine TLR8 polypeptide by eliminating proteolysis by proteases in an expression system (e.g., dibasic amino acid residues in yeast expression systems in which KEX2 protease activity is present).

Mutations of a nucleic acid which encode a murine TLR7 or murine TLR8 polypeptide preferably preserve the amino acid reading frame of the coding sequence, and preferably do not create regions in the nucleic acid which are likely to hybridize to form secondary structures, such as hairpins or loops, which can be deleterious to expression of the variant polypeptide. Methods of making mutations of murine TLR7 or murine TLR8 are as

described elsewhere herein with reference to making mutations of murine TLR9.

The activity of variants of murine TLR7 and murine TLR8 polypeptides can be tested by cloning the gene encoding the variant murine TLR7 or murine TLR8 polypeptide into a prokaryotic or eukaryotic (e.g., mammalian) expression vector, introducing the vector into an appropriate host cell, expressing the variant murine TLR7 or murine TLR8 polypeptide, and testing for a functional capability of the murine TLR7 or murine TLR8 polypeptides as disclosed herein.

The skilled artisan will also realize that conservative amino acid substitutions may be made in murine TLR7 and murine TLR8 polypeptides to provide functionally equivalent variants of the foregoing polypeptides, i.e., variants which retain the functional capabilities of the murine TLR7 and murine TLR8 polypeptides.

A variety of methodologies well known to the skilled practitioner can be utilized to obtain isolated murine TLR7 and murine TLR8 polypeptide molecules, as previously described in reference to murine TLR9 polypeptides.

The invention as described herein has a number of uses, some of which are described elsewhere herein. For example, the invention permits isolation of the murine TLR7 and the murine TLR8 polypeptide molecules by, e.g., expression of a recombinant nucleic acid to produce large quantities of polypeptide which may be isolated using standard protocols. As another example, the isolation of the murine TLR7 gene makes it possible for murine TLR7 to be used in methods for assaying molecular interactions involving TLR7.

The invention also embraces agents which bind selectively to the murine TLR7 or murine TLR8 nucleic acid molecules or polypeptides as well as agents which bind to variants and fragments of the polypeptides and nucleic acids as described herein. The agents include polypeptides which bind to murine TLR7 or murine TLR8, and antisense nucleic acids, both of which are described in greater detail below. Some agents can inhibit or increase murine TLR7-mediated signaling activity (antagonists and agonists, respectively), and some can inhibit or increase murine TLR8-mediated signaling activity.

In one embodiment the murine TLR7 inhibitor is an antisense oligonucleotide that selectively binds to a murine TLR7 nucleic acid molecule, to reduce the expression of murine TLR7 (or TLR7 of another species) in a cell. This is desirable in virtually any medical condition wherein a reduction of TLR7 signaling activity is desirable. Based upon SEQ ID

NO:173 and SEQ ID NO:174, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention.

In one embodiment the murine TLR8 inhibitor is an antisense oligonucleotide that  
5 selectively binds to a murine TLR8 nucleic acid molecule, to reduce the expression of murine TLR8 (or TLR8 of another species) in a cell. This is desirable in virtually any medical condition wherein a reduction of TLR8 signaling activity is desirable. Based upon SEQ ID NO:190 and SEQ ID NO:191, or upon allelic or homologous genomic and/or cDNA sequences, one of skill in the art can easily choose and synthesize any of a number of appropriate antisense molecules for use in accordance with the present invention.  
10

Antisense oligonucleotides for murine TLR7 or murine TLR8 can include "natural" and "modified" oligonucleotides as previously described herein.

Agents which bind murine TLR7 or murine TLR8 also include binding peptides and other molecules which bind to the murine TLR7 or murine TLR8 polypeptide and complexes  
15 containing the murine TLR7 or murine TLR8 polypeptide, respectively. When the binding molecules are inhibitors, the molecules bind to and inhibit the activity of murine TLR7 or murine TLR8. When the binding molecules are activators, the molecules bind to and increase the activity of murine TLR7 or murine TLR8. To determine whether a murine TLR7 or murine TLR8 binding agent binds to murine TLR7 or murine TLR8, any known binding  
20 assay may be employed. For example, the binding agent may be immobilized on a surface and then contacted with a labeled murine TLR7 or murine TLR8 polypeptide. The amount of murine TLR7 or murine TLR8 which interacts with the murine TLR7 or murine TLR8 binding agent, or the amount which does not bind to the murine TLR7 or murine TLR8 binding agent, may then be quantitated to determine whether the murine TLR7 or murine  
25 TLR8 binding agent binds to murine TLR7 or murine TLR8.

The murine TLR7 or murine TLR8 binding agents include molecules of numerous size and type that bind selectively or preferentially to murine TLR7 or murine TLR8 polypeptides, and to complexes involving murine TLR7 or murine TLR8 polypeptides and their binding partners. These molecules may be derived from a variety of sources. For  
30 example, murine TLR7 or murine TLR8 binding agents can be provided by screening degenerate peptide libraries which can be readily prepared in solution, in immobilized form or

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as phage display libraries. Combinatorial libraries also can be synthesized of peptides containing one or more amino acids. Libraries further can be synthesized of peptoids and non-peptide synthetic moieties.

Exemplary methods useful for identifying murine TLR7 and murine TLR8 binding 5 peptides are analogous to those described herein with reference to methods for identifying murine TLR9 binding peptides murine, and thus are not repeated here.

Therefore the invention generally provides efficient methods of identifying pharmacological agents or lead compounds for agents useful in the treatment of conditions associated with TLR7 and TLR8 activity, and the compounds and agents so identified.

10 Generally, the screening methods involve assaying for compounds which inhibit or enhance the expression of or signaling through murine TLR7 or murine TLR8. Such methods are adaptable to automated, high throughput screening of compounds.

A variety of assays for pharmacological agents are provided, including labeled *in vitro* protein binding assays, signaling assays using detectable molecules, etc. For example, protein 15 binding screens are used to rapidly examine the binding of candidate pharmacological agents to a murine TLR7 or murine TLR8. The candidate pharmacological agents can be derived from, for example, combinatorial peptide or nucleic acid libraries. Convenient reagents for such assays are known in the art. An exemplary cell-based assay of signaling involves contacting a cell having a murine TLR7 or murine TLR8 with a candidate pharmacological 20 agent under conditions whereby the induction of a detectable molecule can occur. A reduced degree of induction of the detectable molecule in the presence of the candidate pharmacological agent indicates that the candidate pharmacological agent reduces the signaling activity of murine TLR7 or murine TLR8. An increased degree of induction of the detectable molecule in the presence of the candidate pharmacological agent indicates that the 25 candidate pharmacological agent increases the signaling activity of murine TLR7 or murine TLR8.

Murine TLR7 and murine TLR8 used in the methods of the invention can be added to an assay mixture as an isolated polypeptide (where binding of a candidate pharmaceutical agent is to be measured) or as a cell or other membrane-encapsulated space which includes a 30 murine TLR7 or murine TLR8 polypeptide. In the latter assay configuration, the cell or other membrane-encapsulated space can contain the murine TLR7 or murine TLR8 as a

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polypeptide or as a nucleic acid (e.g., a cell transfected with an expression vector containing a nucleic acid molecule encoding murine TLR7). In the assays described herein, the murine TLR7 or murine TLR8 polypeptide can be produced recombinantly, isolated from biological extracts, or synthesized *in vitro*. Murine TLR7 or murine TLR8 polypeptides encompass 5 chimeric proteins comprising a fusion of a murine TLR7 or murine TLR8 polypeptide with another polypeptide, e.g., a polypeptide capable of providing or enhancing protein-protein binding, enhancing signaling capability, facilitating detection, or enhancing stability of the murine TLR7 or murine TLR8 polypeptide under assay conditions. A polypeptide fused to a murine TLR7 or murine TLR8 polypeptide or fragment thereof may also provide means of 10 readily detecting the fusion protein, e.g., by immunological recognition or by fluorescent labeling.

The assay mixture also comprises a candidate pharmacological agent, as previously described in reference to murine TLR9. Candidate pharmacologic agents are obtained from a wide variety of sources, including libraries of natural, synthetic, or semisynthetic compounds, 15 or any combination thereof. Presently, natural ligands of murine TLR7 and murine TLR8 are unknown, but they appear not to include CpG-ODN.

A variety of other reagents also can be included in the assay mixture. These include reagents such as salts, buffers, neutral proteins (e.g., albumin), detergents, etc. which may be used to facilitate optimal protein-protein and/or protein-nucleic acid binding. Such a reagent 20 may also reduce non-specific or background interactions of the reaction components. Other reagents that improve the efficiency of the assay such as protease inhibitors, nuclease inhibitors, antimicrobial agents, and the like may also be used.

The mixture of the foregoing assay materials is incubated under conditions whereby, but for the presence of the candidate pharmacological agent, the murine TLR7 or murine 25 TLR8 mediates TLR7-mediated or TLR8-mediated signaling, preferably TLR/IL-1R signaling. For determining the binding of a candidate pharmaceutical agent to a murine TLR7 or murine TLR8, the mixture is incubated under conditions which permit binding. The order of addition of components, incubation temperature, time of incubation, and other parameters of the assay may be readily determined. Such experimentation merely involves optimization 30 of the assay parameters, not the fundamental composition of the assay. Incubation temperatures typically are between 4°C and 40°C. Incubation times preferably are minimized

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to facilitate rapid, high throughput screening, and typically are between 1 minute and 10 hours.

After incubation, the level of signaling or the level of specific binding between the murine TLR7 or murine TLR8 polypeptide and the candidate pharmaceutical agent is  
5 detected by any convenient method available to the user, as described elsewhere herein.

The murine TLR7 or murine TLR8 binding agent may also be an antibody or a functionally active antibody fragment. Antibodies, including monoclonal antibodies and antibody fragments, are well known to those of ordinary skill in the science of immunology and are as described elsewhere herein. Monoclonal antibodies may be made by any of the  
10 methods known in the art utilizing murine TLR7 or murine TLR8, or a fragment thereof, as an immunogen. Alternatively the antibody may be a polyclonal antibody specific for murine TLR7 or murine TLR8 which inhibits murine TLR7 or murine TLR8 activity. The preparation and use of polyclonal antibodies are also known to one of ordinary skill in the art.

The sequences of the antigen-binding Fab' portion of the anti-murine TLR7 or anti-murine TLR8 monoclonal antibodies identified as being useful according to the invention in the assays provided above, as well as the relevant FR and CDR regions, can be determined using amino acid sequencing methods that are routine in the art. Such sequence information can be used to generate humanized and chimeric antibodies, as well as various fusion proteins and binding fragments, as described elsewhere herein.  
15

Thus, as will be apparent to one of ordinary skill in the art, the present invention also provides for F(ab')<sub>2</sub> and Fab fragments of an anti-murine TLR7 or anti-murine TLR8 monoclonal antibody; chimeric antibodies in which the Fc and/or FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-murine TLR7 or anti-murine TLR8 antibody have been replaced by homologous human or non-human sequences; chimeric  
25 F(ab')<sub>2</sub> fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions of an anti-murine TLR7 or anti-murine TLR8 antibody have been replaced by homologous human or non-human sequences; and chimeric Fab fragment antibodies in which the FR and/or CDR1 and/or CDR2 and/or light chain CDR3 regions have been replaced by homologous human or non-human sequences.

According to the invention murine TLR7 and murine TLR8 inhibitors also include "dominant negative" polypeptides derived from SEQ ID NO:175 or SEQ ID NO:192,  
30

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respectively. The end result of the expression of a dominant negative murine TLR7 or dominant negative murine TLR8 polypeptide of the invention in a cell is a reduction in TLR7 or murine TLR8 activity such as signaling through the TIR pathway. One of ordinary skill in the art can assess the potential for a dominant negative variant of a murine TLR7 or dominant negative murine TLR8 polypeptide and, using standard mutagenesis techniques, create one or more dominant negative variant polypeptides.

Each of the compositions according to this aspect of the invention is useful for a variety of therapeutic and non-therapeutic purposes. For example, the murine TLR7 and murine TLR8 nucleic acids of the invention are useful as oligonucleotide probes. Such oligonucleotide probes can be used herein to identify genomic or cDNA library clones possessing an identical or substantially similar nucleic acid sequence. Methods of hybridization, synthesis of probes, and detection are generally as described elsewhere herein.

Additionally, complements of the murine TLR7 and murine TLR8 nucleic acids can be useful as antisense oligonucleotides, e.g., by delivering the antisense oligonucleotide to an animal to induce a murine TLR7 or murine TLR8 "knockout" phenotype.

Alternatively, the murine TLR7 and murine TLR8 nucleic acids of the invention can be used to prepare a non-human transgenic animal. The invention, therefore, contemplates the use of murine TLR7 and murine TLR8 knockout and transgenic animals as models for the study of disorders involving TLR7- and murine TLR8-mediated signaling. A variety of methods known to one of ordinary skill in the art are available for the production of transgenic animals associated with this invention.

Inactivation or replacement of the endogenous TLR7 or TLR8 gene can be achieved by a homologous recombination system using embryonic stem cells. The resultant transgenic non-human mammals having a TLR7<sup>-/-</sup> or TLR8<sup>-/-</sup> knockout phenotype may be made transgenic for the murine TLR7 or murine TLR8 and used as a model for screening compounds as modulators (agonists or antagonists/inhibitors) of the murine TLR7 or murine TLR8. In this manner, such therapeutic drugs can be identified.

Additionally, a normal or mutant version of murine TLR7 or murine TLR8 can be inserted into the germ line to produce transgenic animals which constitutively or inducibly express the normal or mutant form of murine TLR7 or murine TLR8. These animals are useful in studies to define the role and function of murine TLR7 or murine TLR8 in cells.

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The antagonists, agonists, nucleic acids, and polypeptides of murine TLR7 and murine TLR8 useful according to the invention may be combined, optionally, with a pharmaceutically acceptable carrier. Thus the invention also provides pharmaceutical compositions and a method for preparing the pharmaceutical compositions which contain 5 compositions of this aspect of the invention. The pharmaceutical compositions include one or any combination of the antagonists, agonists, nucleic acids and polypeptides of murine TLR7 and murine TLR8 useful according to the invention and, optionally, a pharmaceutically acceptable carrier. Each pharmaceutical composition is prepared by selecting an antagonist, agonist, nucleic acid or polypeptide of murine TLR7 and murine TLR8 useful according to 10 the invention, as well as any combination thereof, and, optionally, combining it with a pharmaceutically acceptable carrier.

A variety of administration routes are available, as described previously herein. The particular mode selected will depend, of course, upon the particular compound selected, the severity of the condition being treated, and the dosage required for therapeutic efficacy.

15 Likewise, a variety of formulations are contemplated, including, by analogy those discussed above in reference to murine TLR9, unit dose solids, liquids, extended release formulations, etc.

### Screening Assays

20 In another aspect the invention provides methods for screening candidate compounds that act as ISNA mimics, agonists or antagonists in ISNA-induced immunomodulation via TLR7, TLR8, and TLR9. Preferably the screening method can be adapted to accommodate high throughput screening assays, as can be achieved, for example, through the use of multiwell arrays of samples in conjunction with robotic or automated array handling devices.

25 Immunostimulatory nucleic acids include but are not limited to CpG nucleic acids.

A “CpG nucleic acid” or a “CpG immunostimulatory nucleic acid” as used herein is a nucleic acid containing at least one unmethylated CpG dinucleotide (cytosine-guanine dinucleotide sequence, i.e. “CpG DNA” or DNA containing a 5' cytosine followed by 3' guanine and linked by a phosphate bond) and activates a component of the immune system.

30 The entire CpG nucleic acid can be unmethylated or portions may be unmethylated but at least the C of the 5' CG 3' must be unmethylated.

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In one embodiment a CpG nucleic acid is represented by at least the formula:



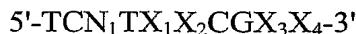
wherein X<sub>1</sub> and X<sub>2</sub> are nucleotides, N is any nucleotide, and N<sub>1</sub> and N<sub>2</sub> are nucleic acid sequences composed of from about 0-25 N's each. In some embodiments X<sub>1</sub> is adenine, 5 guanine, or thymine and/or X<sub>2</sub> is cytosine, adenine, or thymine. In other embodiments X<sub>1</sub> is cytosine and/or X<sub>2</sub> is guanine.

In other embodiments the CpG nucleic acid is represented by at least the formula:



wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> are nucleotides; N is any nucleotide; and N<sub>1</sub> and N<sub>2</sub> are nucleic acid sequences composed of from about 0-25 N's each. In some embodiments, X<sub>1</sub>X<sub>2</sub> are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X<sub>3</sub>X<sub>4</sub> are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA. In some 10 embodiments, X<sub>1</sub>X<sub>2</sub> are GpA or GpT and X<sub>3</sub>X<sub>4</sub> are TpT. In other embodiments X<sub>1</sub> or X<sub>2</sub> or both are purines and X<sub>3</sub> or X<sub>4</sub> or both are pyrimidines or X<sub>1</sub>X<sub>2</sub> are GpA and X<sub>3</sub> or X<sub>4</sub> or both 15 are pyrimidines.

In another embodiment the CpG nucleic acid is represented by at least the formula:



wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> are nucleotides; N is any nucleotide; and N<sub>1</sub> and N<sub>2</sub> are nucleic acid sequences composed of from about 0-25 N's each. In some embodiments, X<sub>1</sub>X<sub>2</sub> are nucleotides selected from the group consisting of: GpT, GpG, GpA, ApA, ApT, ApG, CpT, CpA, CpG, TpA, TpT, and TpG; and X<sub>3</sub>X<sub>4</sub> are nucleotides selected from the group consisting of: TpT, CpT, ApT, TpG, ApG, CpG, TpC, ApC, CpC, TpA, ApA, and CpA. In some 20 embodiments, X<sub>1</sub>X<sub>2</sub> are GpA or GpT and X<sub>3</sub>X<sub>4</sub> are TpT. In other embodiments X<sub>1</sub> or X<sub>2</sub> or both are purines and X<sub>3</sub> or X<sub>4</sub> or both are pyrimidines or X<sub>1</sub>X<sub>2</sub> are GpA and X<sub>3</sub> or X<sub>4</sub> or both 25 are pyrimidines.

Examples of CpG nucleic acids according to the invention include but are not limited to those listed in Table 1, such as SEQ ID NOs:21-29, 31-42, 44, 46-50, 52-62, 64-75, 77-88, 90-117, 119-124.

**Table 1. Exemplary CpG nucleic acids**

	AACGTTCT	SEQ ID NO:21
	AAGCGAAAATGAAATTGACT	SEQ ID NO:22
5	ACCATGGACGA <u>CTGTTCCCCTC</u>	SEQ ID NO:23
	ACCATGGAC <u>GACCTGTTCCCCTC</u>	SEQ ID NO:24
	ACCATGGAC <u>GAGCTGTTCCCCTC</u>	SEQ ID NO:25
	ACCATGGAC <u>GGATCTGTTCCCCTC</u>	SEQ ID NO:26
	ACCATGGAC <u>GGGTCTGTTCCCCTC</u>	SEQ ID NO:27
10	ACCATGGAC <u>GCTACTGTTCCCCTC</u>	SEQ ID NO:28
	ACCATGGAC <u>GCTCTGTTCCCCTC</u>	SEQ ID NO:29
	AGATTTCTAGGAATTCAATC	SEQ ID NO:30
	AGCGGGGGCGAGCGGGGCG	SEQ ID NO:31
	AGCTATGAC <u>GTTCCAAGG</u>	SEQ ID NO:32
15	ATCGACT <u>CTCGAGCGTCTC</u>	SEQ ID NO:33
	ATGAC <u>GTTCTGACGTT</u>	SEQ ID NO:34
	ATGGAAGGT <u>CCAACGTTCTC</u>	SEQ ID NO:35
	ATGGAAGGT <u>CCAGCGTCTC</u>	SEQ ID NO:36
	ATGGACT <u>CTCCAGCGTCTC</u>	SEQ ID NO:37
20	ATGGAGG <u>GTCATCGTTCTC</u>	SEQ ID NO:38
	CAA <u>CGTT</u>	SEQ ID NO:39
	CACGTT <u>GAGGGGCAT</u>	SEQ ID NO:40
	CAGGCATA <u>ACCGTTCCGT</u>	SEQ ID NO:41
	CCA <u>ACGTT</u>	SEQ ID NO:42
25	CTCCTAGT <u>GGGGGTGTCCTAT</u>	SEQ ID NO:43
	CTGAT <u>TTCCCCGAAATGATG</u>	SEQ ID NO:44
	CTGCT <u>GAGACTGGAG</u>	SEQ ID NO:45
	GAGAACGAT <u>GGCACCTCCAT</u>	SEQ ID NO:46
	GAGAACG <u>CTCCAGCACTGAT</u>	SEQ ID NO:47
30	GAGAACG <u>CTCGACCTTCAT</u>	SEQ ID NO:48
	GAGAAC <u>GCTGGACCTTCAT</u>	SEQ ID NO:49
	GAGCAAG <u>GCTGGACCTTCAT</u>	SEQ ID NO:50
	GATTGC <u>CTGACGTCAAGAGAG</u>	SEQ ID NO:51
	GCATGAC <u>GTTGAGCT</u>	SEQ ID NO:52
35	GC <u>GGCGGGGGCGCGCGGCC</u>	SEQ ID NO:53
	GC <u>GTGCGTTGTCGTTGCGTT</u>	SEQ ID NO:54
	GCT <u>AGACGTTAGCGT</u>	SEQ ID NO:55
	GCT <u>AGACGTTAGTGT</u>	SEQ ID NO:56
40	GCT <u>AGATGTTAGCGT</u>	SEQ ID NO:57
	GCT <u>TGATGACTCAGCCGAA</u>	SEQ ID NO:58
	GG <u>ATGACGTTCCGTG</u>	SEQ ID NO:59
	GG <u>GGTCAACGTTGACGGG</u>	SEQ ID NO:60
	GG <u>GGTCACTGACGGG</u>	SEQ ID NO:61
	GG <u>GGTCACTGACGGG</u>	SEQ ID NO:62
45	GT <u>ATTTCCCAGAAAAGAAC</u>	SEQ ID NO:63
	GT <u>CCATTTC<u>CCGTAAATCTT</u></u>	SEQ ID NO:64
	GT <u>CGCT</u>	SEQ ID NO:65
	GT <u>CGTT</u>	SEQ ID NO:66
	TAC <u>CGCGTGC<u>GCACCCCTC</u></u>	SEQ ID NO:67
50	TAT <u>GCATATT<u>CCGTAAAGTG</u></u>	SEQ ID NO:68
	TCA <u>ACGTC</u>	SEQ ID NO:69
	TCA <u>ACGTT</u>	SEQ ID NO:70
	TCA <u>AGCTT</u>	SEQ ID NO:71
	TC <u>AGCGCT</u>	SEQ ID NO:72
55	TC <u>AGCGTGC<u>GCC</u></u>	SEQ ID NO:73
	TC <u>ATCGAT</u>	SEQ ID NO:74
	TCC <u>ACGACGTT<u>TCGACGTT</u></u>	SEQ ID NO:75
	TCC <u>AGGACTT<u>CTCTCAGGTT</u></u>	SEQ ID NO:76
	TCC <u>ATAACGTT<u>CCTGATGCT</u></u>	SEQ ID NO:77
60	TCC <u>ATAGCGT<u>CCCTAGCGTT</u></u>	SEQ ID NO:78
	TCC <u>CATCACG<u>TGCCTGATGCT</u></u>	SEQ ID NO:79
	TCC <u>CATGAC<u>GGTCCTGATGCT</u></u>	SEQ ID NO:80
	TCC <u>CATGAC<u>GTCCCTGATGCT</u></u>	SEQ ID NO:81

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	TCCATGACGTGCCTGATGCT	SEQ ID NO: 82
	TCCATGACGTTCCCTGACGTT	SEQ ID NO: 83
	TCCATGACGTTCCCTGATGCT	SEQ ID NO: 84
	TCCATGAGCTTCCTGATGCT	SEQ ID NO: 85
5	TCCATGCCGGTCCCTGATGCT	SEQ ID NO: 86
	TCCATGCGTGCCTGCGTTTT	SEQ ID NO: 87
	TCCATGCGTGCCTGCGTT	SEQ ID NO: 88
	TCCATGCTGGTCCCTGATGCT	SEQ ID NO: 89
	TCCATGGCCGTCCTGATGCT	SEQ ID NO: 90
10	TCCATGTCGATCCTGATGCT	SEQ ID NO: 91
	TCCATGTCGCTCCTGATGCT	SEQ ID NO: 92
	TCCATGTCGGTCCCTGATGCT	SEQ ID NO: 93
	TCCATGTCGGTCCCTGCTGAT	SEQ ID NO: 94
15	TCCATGTCGTCCTGATGCT	SEQ ID NO: 95
	TCCATGTCGTTCCCTGATGCT	SEQ ID NO: 96
	TCCATGTCGTTCCCTGTCGTT	SEQ ID NO: 97
	TCCATGTCGTTTTGTCGTT	SEQ ID NO: 98
	TCCGACGTTCCCTGACGTT	SEQ ID NO: 99
20	TCCGTCGTTCCCTGTCGTT	SEQ ID NO: 100
	TCCGTCGTTCCCTGTCGTT	SEQ ID NO: 101
	TCCGTCGTTTTGTCGTT	SEQ ID NO: 102
	TCCGTCGTTCCCTGTCGTT	SEQ ID NO: 103
	<u>TCGATCGGGGCGGGGCGAGC</u>	SEQ ID NO: 104
25	<u>TCGTCGCTGTCCTCCGCTTCTT</u>	SEQ ID NO: 105
	TCGTCGCTGTCCTCCGCTTCTT	SEQ ID NO: 106
	TCGTCGCTGTCCTGCCCTTCTT	SEQ ID NO: 107
	TCGTCGCTGTTGTCGTTCTT	SEQ ID NO: 108
	<u>TCGTCGTCGTCGTT</u>	SEQ ID NO: 109
30	TCGTCGTTGTCGTTGTCGTT	SEQ ID NO: 110
	TCGTCGTTGTCGTTTGTCGTT	SEQ ID NO: 111
	<u>TCGTCGTTTGTCGTTTGTCGTT</u>	SEQ ID NO: 112
	<u>TCTCCCAGCGCGCCAT</u>	SEQ ID NO: 113
	TCTCCCAGCGGGCGCAT	SEQ ID NO: 114
35	TCTCCCAGCGTGCGCCAT	SEQ ID NO: 115
	TCTTCGAA	SEQ ID NO: 116
	TGCAGATTGCGCAATCTGCA	SEQ ID NO: 117
	TGCTGCTTTGTCGTTTGTCGCTT	SEQ ID NO: 118
	<u>TGTCGCT</u>	SEQ ID NO: 119
40	<u>TGTCGTT</u>	SEQ ID NO: 120
	<u>TGTCGTTGTCGTT</u>	SEQ ID NO: 121
	<u>TGTCGTTGTCGTTGTCGTT</u>	SEQ ID NO: 122
	TGTCGTTGTCGTTGTCGTT	SEQ ID NO: 123
	<u>TGTCGTTGTCGTTGTCGTT</u>	SEQ ID NO: 124

45 Other ISNAs include but are not limited to T-rich nucleic acids, poly G nucleic acids, and nucleic acids having phosphate modified backbones, such as phosphorothioate backbones.

A "T rich nucleic acid" or "T rich immunostimulatory nucleic acid" is a nucleic acid which includes at least one poly T sequence and/or which has a nucleotide composition of 50 greater than 25% T nucleotide residues and which activates a component of the immune system. A nucleic acid having a poly-T sequence includes at least four Ts in a row, such as 5'TTTT3'. Preferably the T rich nucleic acid includes more than one poly T sequence. In preferred embodiments the T rich nucleic acid may have 2, 3, 4, etc poly T sequences. One of

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the most highly immunostimulatory T rich oligonucleotides discovered according to the invention is a nucleic acid composed entirely of T nucleotide residues. Other T rich nucleic acids have a nucleotide composition of greater than 25% T nucleotide residues, but do not necessarily include a poly T sequence. In these T rich nucleic acids the T nucleotide residues  
5 may be separated from one another by other types of nucleotide residues, i.e., G, C, and A. In some embodiments the T rich nucleic acids have a nucleotide composition of greater than 30%, 40%, 50%, 60%, 70%, 80%, 90%, and 99%, T nucleotide residues and every integer % in between. Preferably the T rich nucleic acids have at least one poly T sequence and a nucleotide composition of greater than 25% T nucleotide residues.

10 In one embodiment the T rich nucleic acid is represented by at least the formula:



wherein  $X_1$ ,  $X_2$ ,  $X_3$ , and  $X_4$  are nucleotides. In one embodiment  $X_1X_2$  is TT and/or  $X_3X_4$  is TT. In another embodiment  $X_1X_2$  are any one of the following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC; and  $X_3X_4$  are any one of the  
15 following nucleotides TA, TG, TC, AT, AA, AG, AC, CT, CC, CA, CG, GT, GG, GA, and GC.

In some embodiments it is preferred that the T-rich nucleic acid does not contain poly C (CCCC), poly A (AAAA), poly G (GGGG), CpG motifs, or multiple GGs. In other embodiments the T-rich nucleic acid includes these motifs. Thus in some embodiments of  
20 the invention the T rich nucleic acids include CpG dinucleotides and in other embodiments the T rich nucleic acids are free of CpG dinucleotides. The CpG dinucleotides may be methylated or unmethylated.

Poly G containing nucleic acids are also immunostimulatory. A variety of references, including Pisetsky and Reich, 1993 *Mol. Biol. Reports*, 18:217-221; Krieger and Herz, 1994,  
25 *Ann. Rev. Biochem.*, 63:601-637; Macaya et al., 1993, *PNAS*, 90:3745-3749; Wyatt et al., 1994, *PNAS*, 91:1356-1360; Rando and Hogan, 1998, In Applied Antisense Oligonucleotide Technology, ed. Krieg and Stein, p. 335-352; and Kimura et al., 1994, *J. Biochem.* 116, 991-994 also describe the immunostimulatory properties of poly G nucleic acids.

Poly G nucleic acids preferably are nucleic acids having the following formulas:

wherein X<sub>1</sub>, X<sub>2</sub>, X<sub>3</sub>, and X<sub>4</sub> are nucleotides. In preferred embodiments at least one of X<sub>3</sub> and X<sub>4</sub> are a G. In other embodiments both of X<sub>3</sub> and X<sub>4</sub> are a G. In yet other embodiments the preferred formula is 5' GGGNGGG 3', or 5' GGGNGGGNGGG 3' wherein N represents between 0 and 20 nucleotides. In other embodiments the Poly G nucleic acid is free of 5 unmethylated CG dinucleotides. In other embodiments the poly G nucleic acid includes at least one unmethylated CG dinucleotide.

Nucleic acids having modified backbones, such as phosphorothioate backbones, also fall within the class of immunostimulatory nucleic acids. U.S. Patents Nos. 5,723,335 and 5,663,153 issued to Hutcherson, et al. and related PCT publication WO95/26204 describe 10 immune stimulation using phosphorothioate oligonucleotide analogues. These patents describe the ability of the phosphorothioate backbone to stimulate an immune response in a non-sequence specific manner.

The ISNAs may be double-stranded or single-stranded. Generally, double-stranded molecules may be more stable *in vivo*, while single-stranded molecules may have increased 15 activity. The terms "nucleic acid" and "oligonucleotide" refer to multiple nucleotides (i.e. molecules comprising a sugar (e.g. ribose or deoxyribose) linked to a phosphate group and to an exchangeable organic base, which is either a substituted pyrimidine (e.g. cytosine (C), thymine (T) or uracil (U)) or a substituted purine (e.g., adenine (A) or guanine (G)) or a modified base. As used herein, the terms refer to oligoribonucleotides as well as 20 oligodeoxyribonucleotides. The terms shall also include polynucleosides (i.e., a polynucleotide minus the phosphate) and any other organic base-containing polymer. The terms "nucleic acid" and "oligonucleotide" also encompass nucleic acids or oligonucleotides with a covalently modified base and/or sugar. For example, they include nucleic acids having 25 backbone sugars which are covalently attached to low molecular weight organic groups other than a hydroxyl group at the 3' position and other than a phosphate group at the 5' position. Thus modified nucleic acids may include a 2'-O-alkylated ribose group. In addition, modified nucleic acids may include sugars such as arabinose instead of ribose. Thus the nucleic acids may be heterogeneous in backbone composition thereby containing any possible combination 30 of polymer units linked together such as peptide- nucleic acids (which have amino acid backbone with nucleic acid bases). In some embodiments the nucleic acids are homogeneous in backbone composition.

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The substituted purines and pyrimidines of the ISNAs include standard purines and pyrimidines such as cytosine as well as base analogs such as C-5 propyne substituted bases. Wagner RW et al., *Nat Biotechnol* 14:840-844 (1996). Purines and pyrimidines include but are not limited to adenine, cytosine, guanine, thymine, 5-methylcytosine, 2-aminopurine, 2-  
5 amino-6-chloropurine, 2,6-diaminopurine, hypoxanthine, and other naturally and non-naturally occurring nucleobases, substituted and unsubstituted aromatic moieties.

The ISNA is a linked polymer of bases or nucleotides. As used herein with respect to linked units of a nucleic acid, "linked" or "linkage" means two entities are bound to one another by any physicochemical means. Any linkage known to those of ordinary skill in the art, covalent or non-covalent, is embraced. Such linkages are well known to those of ordinary skill in the art. Natural linkages, which are those ordinarily found in nature connecting the individual units of a nucleic acid, are most common. The individual units of a nucleic acid may be linked, however, by synthetic or modified linkages.

Whenever a nucleic acid is represented by a sequence of letters it will be understood that the nucleotides are in 5' to 3' order from left to right and that "A" denotes adenine, "C" denotes cytosine, "G" denotes guanine, "T" denotes thymidine, and "U" denotes uracil unless otherwise noted.

Immunostimulatory nucleic acid molecules useful according to the invention can be obtained from natural nucleic acid sources (e.g., genomic nuclear or mitochondrial DNA or cDNA), or are synthetic (e.g., produced by oligonucleotide synthesis). Nucleic acids isolated from existing nucleic acid sources are referred to herein as native, natural, or isolated nucleic acids. The nucleic acids useful according to the invention may be isolated from any source, including eukaryotic sources, prokaryotic sources, nuclear DNA, mitochondrial DNA, etc. Thus, the term nucleic acid encompasses both synthetic and isolated nucleic acids.

The term "isolated" as used herein with reference to an ISNA means substantially free of or separated from components which it is normally associated with in nature, e.g., nucleic acids, proteins, lipids, carbohydrates or *in vivo* systems to an extent practical and appropriate for its intended use. In particular, the nucleic acids are sufficiently pure and are sufficiently free from other biological constituents of host cells so as to be useful in, for example, producing pharmaceutical preparations. Because an isolated nucleic acid of the invention may be admixed with a pharmaceutically-acceptable carrier in a pharmaceutical preparation,

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the nucleic acid may comprise only a small percentage by weight of the preparation. The nucleic acid is nonetheless substantially pure in that it has been substantially separated from the substances with which it may be associated in living systems.

The ISNAs can be produced on a large scale in plasmids, (see *Molecular Cloning: A Laboratory Manual*, J. Sambrook, et al., eds., Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989) and separated into smaller pieces or administered whole. After being administered to a subject the plasmid can be degraded into oligonucleotides. One skilled in the art can purify viral, bacterial, eukaryotic, etc. nucleic acids using standard techniques, such as those employing restriction enzymes, exonucleases or endonucleases.

For use in the instant invention, the ISNAs can be synthesized *de novo* using any of a number of procedures well known in the art. For example, the β-cyanoethyl phosphoramidite method (Beaucage SL and Caruthers MH, *Tetrahedron Lett* 22:1859 (1981)); nucleoside H-phosphonate method (Garegg et al., *Tetrahedron Lett* 27:4051-4054 (1986); Froehler et al., *Nucl Acid Res* 14:5399-5407 (1986); Garegg et al., *Tetrahedron Lett* 27:4055-4058 (1986); Gaffney et al., *Tetrahedron Lett* 29:2619-2622 (1988)). These chemistries can be performed by a variety of automated oligonucleotide synthesizers available in the market.

ISNAs having modified backbones, such as phosphorothioate backbones, also fall within the class of immunostimulatory nucleic acids. U.S. Patents Nos. 5,723,335 and 5,663,153 issued to Hutcherson, et al. and related PCT publication WO95/26204 describe immune stimulation using phosphorothioate oligonucleotide analogues. These patents describe the ability of the phosphorothioate backbone to stimulate an immune response in a non-sequence specific manner.

The ISNA may be any size of at least 6 nucleotides but in some embodiments are in the range of between 6 and 100 or in some embodiments between 8 and 35 nucleotides in size. Immunostimulatory nucleic acids can be produced on a large scale in plasmids. These may be administered in plasmid form or alternatively they can be degraded into oligonucleotides before administration.

“Palindromic sequence” shall mean an inverted repeat (i.e., a sequence such as ABCDEE'D'C'B'A' in which A and A', B and B', etc., are bases capable of forming the usual Watson-Crick base pairs and which includes at least 6 nucleotides in the palindrome. *In vivo*,

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such sequences may form double-stranded structures. In one embodiment the nucleic acid contains a palindromic sequence. In some embodiments when the nucleic acid is a CpG nucleic acid, a palindromic sequence used in this context refers to a palindrome in which the CpG is part of the palindrome, and optionally is the center of the palindrome. In another 5 embodiment the nucleic acid is free of a palindrome. A nucleic acid that is free of a palindrome does not have any regions of 6 nucleotides or greater in length which are palindromic. A nucleic acid that is free of a palindrome can include a region of less than 6 nucleotides which are palindromic.

A “stabilized ISNA” shall mean a nucleic acid molecule that is relatively resistant to 10 *in vivo* degradation (e.g. via an exo- or endo-nuclease). Stabilization can be a function of length or secondary structure. Nucleic acids that are tens to hundreds of kbs long are relatively resistant to *in vivo* degradation. For shorter nucleic acids, secondary structure can stabilize and increase their effect. For example, if the 3' end of an oligonucleotide has self-complementarity to an upstream region, so that it can fold back and form a sort of stem loop 15 structure, then the oligonucleotide becomes stabilized and therefore exhibits more activity.

Some stabilized ISNAs of the instant invention have a modified backbone. It has been demonstrated that modification of the oligonucleotide backbone provides enhanced activity of the ISNAs when administered *in vivo*. Nucleic acids, including at least two phosphorothioate linkages at the 5' end of the oligonucleotide and multiple phosphorothioate linkages at the 3' 20 end, preferably 5, may provide maximal activity and protect the oligonucleotide from degradation by intracellular exo- and endo-nucleases. Other modified oligonucleotides include phosphodiester modified oligonucleotide, combinations of phosphodiester and phosphorothioate oligonucleotide, methylphosphonate, methylphosphorothioate, phosphorodithioate, and combinations thereof. Each of these combinations and their 25 particular effects on immune cells is discussed in more detail in U.S. Patent Nos. 6,194,388 and 6,207,646, the entire contents of which is hereby incorporated by reference. It is believed that these modified oligonucleotides may show more stimulatory activity due to enhanced nuclease resistance, increased cellular uptake, increased protein binding, and/or altered 30 intracellular localization. Both phosphorothioate and phosphodiester nucleic acids are active in immune cells.

Other stabilized ISNAs include: nonionic DNA analogs, such as alkyl- and aryl-

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phosphates (in which the charged phosphonate oxygen is replaced by an alkyl or aryl group), phosphodiester and alkylphosphotriesters, in which the charged oxygen moiety is alkylated. Oligonucleotides which contain diol, such as tetraethyleneglycol or hexaethyleneglycol, at either or both termini have also been shown to be substantially resistant to nuclease degradation.

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For use *in vivo*, ISNAs are preferably relatively resistant to degradation (e.g., via endo- and exo-nucleases). Secondary structures, such as stem loops, can stabilize nucleic acids against degradation. Alternatively, nucleic acid stabilization can be accomplished via phosphate backbone modifications. One type of stabilized nucleic acid has at least a partial phosphorothioate modified backbone. Phosphorothioates may be synthesized using automated techniques employing either phosphoramidate or H-phosphonate chemistries.

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Aryl- and alkyl-phosphonates can be made, e.g., as described in U.S. Patent No. 4,469,863; and alkylphosphotriesters (in which the charged oxygen moiety is alkylated as described in U.S. Patent No. 5,023,243 and European Patent No. 092,574) can be prepared by automated 15 solid phase synthesis using commercially available reagents. Methods for making other DNA backbone modifications and substitutions have been described. Uhlmann E and Peyman A, *Chem Rev* 90:544 (1990); Goodchild J, *Bioconjugate Chem* 1:165 (1990).

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Other sources of immunostimulatory nucleic acids useful according to the invention include standard viral and bacterial vectors, many of which are commercially available. In its broadest sense, a "vector" is any nucleic acid material which is ordinarily used to deliver and facilitate the transfer of nucleic acids to cells. The vector as used herein may be an empty vector or a vector carrying a gene which can be expressed. In the case when the vector is carrying a gene the vector generally transports the gene to the target cells with reduced degradation relative to the extent of degradation that would result in the absence of the vector. 20 In this case the vector optionally includes gene expression sequences to enhance expression of the gene in target cells such as immune cells, but it is not required that the gene be expressed in the cell.

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A basis for certain of the screening assays is the presence of a functional TLR 7, TLR 8, or TLR9 in a cell. The functional TLR in some instances is naturally expressed by the cell. 30 In other instances, expression of the functional TLR can involve introduction or reconstitution of a species-specific TLR9 into a cell or cell line that otherwise lacks the TLR

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or lacks responsiveness to ISNA, resulting in a cell or cell line capable of activating the TLR/IL-1R signaling pathway in response to contact with an ISNA. Examples of cell lines lacking TLR9 or ISNA responsiveness include, but are not limited to, 293 fibroblasts (ATCC CRL-1573), MonoMac-6, THP-1, U937, CHO, and any TLR9 knock-out. The introduction 5 of the species-specific TLR into the cell or cell line is preferably accomplished by transient or stable transfection of the cell or cell line with a TLR-encoding nucleic acid sequence operatively linked to a gene expression sequence (as described above).

The species-specific TLR, including TLR7, TLR8, and TLR9, is not limited to a murine TLR, but rather can include a TLR derived from murine or non-murine sources.

10 Examples of non-murine sources include, but are not limited to, human, bovine, canine, feline, ovine, porcine, and equine. Other species include chicken and fish, e.g., aquaculture species.

15 The species-specific TLR, including TLR7, TLR8, and TLR9, also is not limited to native TLR polypeptides. In certain embodiments the TLR can be, e.g., a chimeric TLR in which the extracellular domain and the cytoplasmic domains are derived from TLR polypeptides from different species. Such chimeric TLR polypeptides, as described above, can include, for example, a human TLR extracellular domain and a murine TLR cytoplasmic domain, each domain derived from the corresponding TLR7, TLR8, or TLR9 of each species.

20 In alternative embodiments, such chimeric TLR polypeptides can include chimeras created with different TLR splice variants or allotypes. Other chimeric TLR polypeptides useful for the purposes of screening ISNA mimics, agonists and antagonists can include chimeric polypeptides created with a TLR of a first type, e.g., TLR9, and another TLR, e.g., TLR7 or TLR8, of the same or another species as the TLR of the first type. Also contemplated are 25 chimeric polypeptides which incorporate sequences derived from more than two polypeptides, e.g., an extracellular domain, a transmembrane domain, and a cytoplasmic domain all derived from different polypeptide sources, provided at least one such domain derives from a TLR7, TLR8, or TLR9 polypeptide. As a further example, also contemplated are constructs such as include an extracellular domain of one TLR9, an intracellular domain of another TLR9, and a non-TLR reporter such as luciferase, GFP, etc. Those of skill in the art will recognize how to 30 design and generate DNA sequences coding for such chimeric TLR polypeptides.

The screening assays can have any of a number of possible readout systems based

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upon either TLR/IL-1R signaling pathway or other assays useful for assaying response to ISNAs. It has been reported that immune cell activation by CpG immunostimulatory sequences is dependent in some way on endosomal processing. It is not yet known whether TLR9 is directly involved in this endosomal pathway, or if there is some intermediary  
5 between TLR9 and the endosome.

In preferred embodiments, the readout for the screening assay is based on the use of native genes or, alternatively, cotransfected or otherwise co-introduced reporter gene constructs which are responsive to the TLR/IL-1R signal transduction pathway involving MyD88, TRAF6, p38, and/or ERK. Häcker H et al., *EMBO J* 18:6973-6982 (1999). These pathways activate kinases including κB kinase complex and c-Jun N-terminal kinases. Thus reporter genes and reporter gene constructs particularly useful for the assays can include a reporter gene operatively linked to a promoter sensitive to NF-κB. Examples of such promoters include, without limitation, those for NF-κB, IL-1 $\beta$ , IL-6, IL-8, IL-12 p40, CD80, CD86, and TNF- $\alpha$ . The reporter gene operatively linked to the TLR7-, TLR8-, or TLR9-  
10 sensitive promoter can include, without limitation, an enzyme (e.g., luciferase, alkaline phosphatase,  $\beta$ -galactosidase, chloramphenicol acetyltransferase (CAT), etc.), a bioluminescence marker (e.g., green-fluorescent protein (GFP, U.S. patent 5,491,084), etc.), a surface-expressed molecule (e.g., CD25), and a secreted molecule (e.g., IL-8, IL-12 p40, TNF- $\alpha$ ). In preferred embodiments the reporter is selected from IL-8, TNF- $\alpha$ , NF-κB-  
15 luciferase (NF-κB-luc; Häcker H et al., *EMBO J* 18:6973-6982 (1999)), IL-12 p40-luc (Murphy TL et al., *Mol Cell Biol* 15:5258-5267 (1995)), and TNF-luc (Häcker H et al., *EMBO J* 18:6973-6982 (1999)). In assays relying on enzyme activity readout, substrate can be supplied as part of the assay, and detection can involve measurement of chemiluminescence, fluorescence, color development, incorporation of radioactive label, drug  
20 resistance, or other marker of enzyme activity. For assays relying on surface expression of a molecule, detection can be accomplished using FACS analysis or functional assays. Secreted molecules can be assayed using enzyme-linked immunosorbent assay (ELISA) or bioassays.  
25 Many such readout systems are well known in the art and are commercially available.

In another aspect the invention provides a screening method for identifying an immunostimulatory nucleic acid molecule (ISNA). The method entails contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a test  
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nucleic acid molecule; detecting the presence or absence of a response mediated by a TLR signal transduction pathway in the presence of the test nucleic acid molecule arising as a result of an interaction between the functional TLR and the test nucleic acid molecule; and determining the test nucleic acid molecule is an ISNA when the presence of a response

- 5 mediated by the TLR signal transduction pathway is detected. "Functional TLR" and a "cell expressing functional TLR" are as described elsewhere herein. A response mediated by a TLR signal transduction pathway includes induction of a gene under control of a promoter responsive to the TLR/IL-1R signaling pathway, including but not limited to promoters responsive to NF- $\kappa$ B. The biological response thus can include, e.g., secretion of IL-8 and
- 10 luciferase activity in a cell transfected with NF- $\kappa$ B-luc, IL-12 p40-luc, or TNF-luc. A test nucleic acid molecule can include a DNA, RNA, or modified nucleic acid molecule as described herein. In some embodiments the test nucleic acid molecule is a CpG nucleic acid.

Preferably, the test nucleic acid molecule is a sequence variant of a reference ISNA, containing at least one alternative base, at least one alternative internucleotide backbone linkage, or at least one alternative sugar moiety as compared to the particular reference ISNA. In a preferred embodiment the test nucleic acid molecule is a member of a library of such test nucleic acid molecules.

- According to one embodiment of this method, comparison can be made to a reference ISNA. The reference ISNA may be any ISNA, including a CpG nucleic acid. In preferred 20 embodiments the screening method is performed using a plurality of test nucleic acids. Preferably comparison of test and reference responses is based on comparison of quantitative measurements of responses in each instance.

The method can be used to select a subset of test nucleic acid molecules based on their ability to induce a similar specific response mediated by the TLR signal transduction 25 pathway. For instance, the method can be used to classify test CpG nucleic acids as predominantly B-cell activating CpG nucleic acids, or as predominantly IFN- $\alpha$  inducing CpG nucleic acids. Other new classes of ISNAs may be identified and characterized using the method.

Application of this method permits the identification of ISNAs, delineation of 30 sequence specificity of a given TLR, and also optimization of ISNA sequences. Identification of ISNAs involves screening candidate ISNAs as above and selecting any ISNA that induces

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a response as defined. Delineation of sequence specificity involves screening candidate ISNAs as above with reference to a particular TLR9, selecting any ISNAs that induce a response as defined, and categorizing ISNAs that do and do not induce a response on the basis of their sequence. Optimization of ISNA sequences involves an iterative application of  
5 the method as described, further including the steps of selecting the best sequence at any given stage or round in the screening and substituting it as a benchmark or reference in a subsequent round of screening. This latter process can further include selection of parameters to modify in choosing and generating candidate ISNAs to screen.

In another aspect the invention provides screening method for identifying species  
10 specificity of an ISNA. The method involves contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a first species with a test ISNA; contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a second species with the test ISNA; measuring a response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test  
15 ISNA; measuring a response mediated by the TLR signal transduction pathway associated with the contacting the functional TLR of the second species with the test ISNA; and comparing (a) the response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test ISNA with (b) the response mediated by the TLR signal transduction pathway associated with the contacting the  
20 functional TLR of the second species with the test ISNA. The functional TLR may be expressed by a cell or it may be part of a cell-free system. The functional TLR may be part of a complex, with either another TLR or with another protein, e.g., MyD88, IRAK, TRAF6, I $\kappa$ B, NF- $\kappa$ B, or functional homologues and derivatives thereof. Thus for example a given ODN can be tested against a panel of 293 fibroblast cells transfected with TLR7, TLR8, or  
25 TLR9 from various species and optionally cotransfected with a reporter construct (e.g., NF- $\kappa$ B-luc) sensitive to TLR/IL-1R activation pathways. Thus in another aspect, the invention provides a method for screening species selectivity with respect to a given nucleic acid sequence.

As mentioned above, the invention in one aspect provides a screening method for  
30 comparing TLR signaling activity or a test compound against corresponding TLR signaling activity of a reference ISNA. The methods generally involve contacting a functional TLR

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selected from the group consisting of TLR7, TLR8, and TLR9 with a reference ISNA and detecting a reference response mediated by a TLR signal transduction pathway; contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a test compound and detecting a test response mediated by a TLR signal transduction pathway; and

5 comparing the test response with the reference response to compare the TLR signaling activity of the test compound with the ISNA. Assays in which the test compound and the reference ISNA contact the TLR independently may be used to identify test compounds that are ISNA mimics. Assays in which the test compound and the reference ISNA contact the TLR concurrently may be used to identify test compounds that are ISNA agonists and ISNA

10 antagonists.

An ISNA mimic as used herein is a compound which causes a response mediated by a TLR signal transduction pathway. As used herein the term "response mediated by a TLR signal transduction pathway" refers to a response which is characteristic of an ISNA-TLR interaction. As demonstrated herein responses which are characteristic of ISNA-TLR interactions include the induction of a gene under control of an ISNA-specific promoter such as a NF- $\kappa$ B promoter, increases in Th1 cytokine levels, etc. The gene under the control of the NF- $\kappa$ B promoter may be a gene which naturally includes an NF- $\kappa$ B promoter or it may be a gene in a construct in which an NF- $\kappa$ B promoter has been inserted. Genes which naturally include the NF- $\kappa$ B promoter include but are not limited to IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc. Increases in Th1 cytokine levels is another measure characteristic of an ISNA-TLR interaction. Increases in Th1 cytokine levels may result from increased production or increased stability or increased secretion of the Th1 cytokines in response to the ISNA-TLR interaction. Th1 cytokines include but are not limited to IL-2, IFN- $\gamma$ , and IL-12. Other responses which are characteristic of an ISNA-TLR interaction include but are not limited to a reduction in Th2 cytokine levels. Th2 cytokines include but are not limited to IL-4, IL-5, and IL-10.

The response which is characteristic of an ISNA-TLR interaction may be a direct response or an indirect response. A direct response is a response that arises directly as a result of the ISNA-TLR interaction. An indirect response is a response which involves the modulation of other parameters prior to its occurrence.

An ISNA agonist as used herein is a compound which causes an enhanced response to

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an ISNA mediated by a TLR signal transduction pathway. Thus an ISNA agonist as used herein is a compound which causes an increase in at least one aspect of an immune response that is ordinarily induced by the reference ISNA. For example, an immune response that is ordinarily induced by an ISNA can specifically include TLR7-, TLR8-, or TLR9-mediated signal transduction in response to immunostimulatory CpG nucleic acid. An ISNA agonist will in some embodiments compete with ISNA for binding to TLR7, TLR8, or TLR9. In other embodiments an ISNA agonist will bind to a site on TLR7, TLR8, or TLR9 that is distinct from the site for binding ISNA. In yet other embodiments an ISNA agonist will act via another molecule or pathway distinct from TLR7, TLR8, or TLR9.

An ISNA antagonist as used herein is a compound which causes a decreased response to an ISNA mediated by a TLR signal transduction pathway. Thus an ISNA antagonist as used herein is a compound which causes a decrease in at least one aspect of an immune response that is ordinarily induced by the reference ISNA. For example, an immune response that is ordinarily induced by an ISNA can specifically include TLR7-, TLR8-, or TLR9-mediated signal transduction in response to immunostimulatory CpG nucleic acid. An ISNA antagonist will in some embodiments compete with ISNA for binding to TLR7, TLR8, or TLR9. In other embodiments an ISNA antagonist will bind to a site on TLR7, TLR8, or TLR9 that is distinct from the site for binding ISNA. In yet other embodiments an ISNA antagonist will act via another molecule or pathway distinct from TLR7, TLR8, or TLR9.

The screening methods for comparing TLR signaling activity of a test compound with signaling activity of an ISNA involve contacting at least one test compound with a functional TLR selected from TLR7, TLR8, and TLR9 under conditions which, in the absence of a test compound, permit a reference ISNA to induce at least one aspect of an immune response. The functional TLR may be expressed by a cell or it may be part of a cell-free system. A cell expressing a functional TLR is a cell that either naturally expresses the TLR, or is a cell into which has been introduced a TLR expression vector, or is a cell manipulated to express TLR in a manner that allows the TLR to be expressed by the cell and to transduce a signal under conditions which normally permit signal transduction by the signal transducing portion of the TLR. The TLR can be a native TLR or it can be a fragment or variant thereof, as described above. According to these methods, the test compound is contacted with a functional TLR or TLR-expressing cell before, after, or simultaneously with contacting a reference ISNA with

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the functional TLR or TLR-expressing cell. A response of the functional TLR or TLR-expressing cell is measured and compared with the corresponding response that results or would result under the same conditions in the absence of the test compound. Where it is appropriate, the response in the absence of the test compound can be determined as a concurrent or historical control. Examples of such responses include, without limitation, a response mediated through the TLR signal transduction pathway, secretion of a cytokine, cell proliferation, and cell activation. In a preferred embodiment, the measurement of a response involves the detection of IL-8 secretion (e.g., by ELISA). In another preferred embodiment, the measurement of the response involves the detection of luciferase activity (e.g., NF- $\kappa$ B-luc, IL-12 p40-luc, or TNF-luc).

Examples of reference ISNAs include, without limitation, those listed in Table 1 (above). In some preferred embodiments the reference ISNA is a CpG nucleic acid.

Test compounds can include but are not limited to peptide nucleic acids (PNAs), antibodies, polypeptides, carbohydrates, lipids, hormones, and small molecules. Test compounds can further include variants of a reference ISNA incorporating any one or combination of the substitutions described above. Test compounds can be generated as members of a combinatorial library of compounds.

In preferred embodiments, the methods for screening test compounds, test nucleic acid molecules, test ISNAs, and candidate pharmacological agents can be performed on a large scale and with high throughput by incorporating, e.g., an array-based assay system and at least one automated or semi-automated step. For example, the assays can be set up using multiwell plates in which cells are dispensed in individual wells and reagents are added in a systematic manner using a multiwell delivery device suited to the geometry of the multiwell plate. Manual and robotic multiwell delivery devices suitable for use in a high throughput screening assay are well known by those skilled in the art. Each well or array element can be mapped in a one-to-one manner to a particular test condition, such as the test compound. Readouts can also be performed in this multiwell array, preferably using a multiwell plate reader device or the like. Examples of such devices are well known in the art and are available through commercial sources. Sample and reagent handling can be automated to further enhance the throughput capacity of the screening assay, such that dozens, hundreds, thousands, or even millions of parallel assays can be performed in a day or in a week. Fully

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robotic systems are known in the art for applications such as generation and analysis of combinatorial libraries of synthetic compounds. See, for example, U.S. patents 5,443,791 and 5,708,158.

5       The invention will be more fully understood by reference to the following examples. These examples, however, are merely intended to illustrate the embodiments of the invention and are not to be construed to limit the scope of the invention.

### Examples

10      **Example 1. Method of cloning the mouse TLR9**

Alignment of human TLR9 protein sequence with mouse EST database using tfasta yielded 7 hits with mouse EST sequences aa197442, ai451215, aa162495, aw048117, ai463056, aw048548, and aa273731. Two primers were designed that bind to aa197442 EST sequence for use in a RACE-PCR to amplify 5' and 3' ends of the mouse TLR9 cDNA. The library used for the RACE PCR was a mouse spleen marathon-ready cDNA commercially available from Clonetech. A 5' fragment with a length of 1800 bp obtained by this method was cloned into Promega pGEM-T Easy vector. After sequencing of the 5' end, additional primers were designed for amplification of the complete mouse TLR9 cDNA. The primer for the 5' end was obtained from the sequence of the 5' RACE product whereas the primer for the 20 3' end was selected from the mouse EST sequence aa273731.

Three independent PCR reactions were set up using a murine macrophage RAW264.7 (ATCC TIB-71) cDNA as a template, and the resulting amplification products were cloned into the pGEM-T Easy vector. The inserts were fully sequenced, translated into protein and aligned to the human protein sequence. One out of three clones was error-free based on 25 alignment comparison (clone mtlr932e.pep). The cDNA sequence for mTLR9 is SEQ ID NO:1, is presented in Table 2. The ATG start codon occurs at base 40, and a TAG termination codon occurs at base 3136. SEQ ID NO:2 (Table 3), corresponding to bases 40-3135 of SEQ ID NO:1, is the coding region for the polypeptide of SEQ ID NO:3.

30      **Table 2. cDNA Sequence for Murine TLR9 (5' to 3'; SEQ ID NO:1)**

tgtcagaggg	agcctcgaaa	gaatcccca	tctcccaaca	tggttctccg	tcgaaggact	60
ctgcacccct	tgtccctctt	ggtacaggct	gcagtgctgg	ctgagactct	ggccctgggt	120

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	accctgcctg ccttcctacc ctgtgagctg aagcctcatg gcctggtgg a ctgcaattgg	180
	ctgttcctga agtctgtacc ccgtttctct gcggcagcat cctgctccaa catcacccgc	240
	ctctccttga tctccaaccc tatccaccac ctgcacaact ccgacttcgt ccacacctgtcc	300
	aacctgcggc agctgaaccc caagtggAAC tgcacccca ctggccttag cccccctgcac	360
5	ttctcttgcc acatgaccat tgagcccaga accttcctgg ctatgcgtac actggaggag	420
	ctgaacctga gctataatgg tatcaccact gtgccccgac tgcccagctc cctggtaat	480
	ctgagcctga gccacaccaa catcctggtt ctagatgcta acagcctcgc cggcctatac	540
	agcctgcgcg ttctcttcat ggacgggaac tgctactaca agaaccctg cacaggagcg	600
	gtgaagggtga ccccaggcgc ctcctggc ctgagcaatc tcacccatct gtctctgaag	660
10	tataacaacc tcacaaaagggt gccccgccaa ctgccccca gcctggagta ctcctggtg	720
	tcctataacc tcattgtcaa gctggggcct gaagacctgg ccaatctgac ctcccttcga	780
	gtacttgcgtg tgggtggaa ttgcgtcgc tgcgaccatg ccccaatcc ctgtatagaa	840
	tgtggccaaa agtccctcca cctgcaccct gagaccttcc atcacctgag ccatctggaa	900
	ggcctggtgc tgaaggacag ctctctccat acactgaact cttctgggtt ccaaggcttg	960
15	gtcaacccctcggtcgcgaaactttctct atgaaagcat caaccacacc	1020
	aatgcctttc agaacctaacc ccgcctgcgc aagctcaacc tgtccttcaa ttaccgcaag	1080
	aaggatccct ttgcccgcct ccacctggca agttccttca agaacctgggt gtcactgcag	1140
	gagctgaaca tgaacggcat cttctccgc tcgctcaaca agtacacgcg cagatggctg	1200
	gccgatctgc ccaaactcca cactctgcat cttcaaatga acttcatcaa ccaggcacag	1260
20	ctcagcatct ttggcacccctt ccgagccctt cgcttgcgtgg acttgtcaga caatcgcatc	1320
	agtgggcctt caacgcgtgc agaagccacc cctgaagagg cagatgtgc agagcaggag	1380
	gagctgttgt ctgcggatcc tcacccagct ccactgagca cccctgccttc taagaacttc	1440
	atggacaggt gtaagaactt caagttcacc atggacctgt ctggaaacaa cctgggtact	1500
	atcaagccag agatgttgtt caatctctca cgcctccagt gtcttagcct gagccacaac	1560
25	tccattgcac aggctgtcaa tggctctcag ttccctgcgc tgactaatct gcagggtgt	1620
	gacctgtccc ataacaaaact ggacttgcac cactggaaat cgttcagtga gctaccacag	1680
	ttgcaggccc tggacctgag ctacaacagc cagcccttta gcatgaaggg tataggccac	1740
	aatttcagtt ttgtggccca tctgtccatg ctacacagcc ttgcctggc acacaatgac	1800
	attcataaccc gtgtgtccctc acatctcaac agcaactcag tgaggttct tgacttcagc	1860
30	ggcaacggta tggccgcata gttggatgag gggggcctt atctccattt cttccaaggc	1920
	ctgagtggcc tgctgaagct ggacctgtct caaaataacc tgcataatcct ccggcccccag	1980
	aaccttgaca acctcccaa gggcgtgaag ctgctgagcc tccgagacaa ctacctatct	2040
	ttctttaact ggaccagtct gtccttcctg cccacccctgg aagtcctaga cctggcaggc	2100
	aaccagctaa agggccctgac caatggcacc ctgcctaattt gcatccctt ccagaaactg	2160
35	gatgtcagca gcaacagtat cgtctctgtg gtcccagcct tcttcgtct ggccgtcgt	2220
	ctgaaagagg tcaacctcag ccacaacatt ctcaagacgg tggatcgctc ctggtttggg	2280
	cccattgtga tgaacctgac agttcttagac gtgagaagca accctctgca ctgtgcctgt	2340
	ggggcagcct tcgttagactt actgttggag gtgcagacca aggtgcctgg cctggctaat	2400
	ggtgtgaagt gtggcagccc cggccagctg cagggccgtt gcatcttcgc acaggacctg	2460
40	cggctgtgcc tggatgaggt cctcttttgg gactgctttg gcctttact cttggctgt	2520
	gccgtggca tgggtggcctatactgcac catctctgcg gctggacgt ctggtaactgt	2580
	tttcatctgt gcttggcattt gctaccccttgc gcttggccca gccgacgcag cgcccaagct	2640

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	ctccccatg atgccttcgt ggtgttcgat aaggcacaga gcgcagttgc ggactgggtg	2700
	tataacgagc tgcgggtgcg gctggaggag cggcgccgc gcccggccct acgcttgcgt	2760
	ctggaggacc gagattggct gcctggccag acgctctcg agaacctctg ggcttccatc	2820
5	tatggagcc gcaagactct atttgtgcgt gcccacacgg accgcgtcag tggcctcctg	2880
	cgcaccagct tcctgctggc tcagcagcgc ctgttggaaag accgcaagga cgtgggttg	2940
	ttgggtatcc tgcgtccggg tgcccaccgc tcccgctatg tgcgactgcg ccagcgtctc	3000
	tgccgcaga gtgtgtctt ctggcccccag cagcccaacg ggcagggggg cttctggcc	3060
	cagctgagta cagccctgac tagggacaac cgcacttct ataaccagaa cttctgccgg	3120
10	ggacctacag cagaatagct cagagcaaca gctggaaaca gctgcattt catgcctggt	3180
	tcccggatg ctctgcctgc	3200

**Table 3. Coding region for murine TLR9 (SEQ ID NO:2)**

	atggttctcc gtcgaaggac tctgcacccc ttgtccctcc tggtagcaggc tgcagtgcgt	60
	gctgagactc tggccctggg taccctgcct gccttcctac cctgtgagct gaagcctcat	120
15	ggcctgggtgg actgcaattt gctgttcctg aagtctgtac cccgtttctc tgcggcagca	180
	tcctgctcca acatcacccg cctctcccttgc atctccaaacc gtatccacca cctgcacaac	240
	tccgacttcg tccacctgtc caacctgcgg cagctgaacc tcaagtggaa ctgtccaccc	300
	actggccta gccccctgca cttcttgc cacatgacca ttgagcccg aaccttcctg	360
	gctatgcgtt cactggagga gctgaacctg agctataatg gtatcaccac tgtgccccga	420
20	ctgcccagct ccctgggtgaa tctgagcctg agccacacca acatcctggt tctagatgct	480
	aacagcctcg ccggcctata cagcctgcgc gttctttca tggacgggaa ctgctactac	540
	aagaacccct gcacaggagc ggtgaagggtg accccaggcg ccctcctggg cctgagcaat	600
	ctcacccatc tgtctctgaa gtataacaac ctcacaaagg tgccccgcca actgcccccc	660
	agcctggagt acctcctggt gtcctataac ctcattgtca agctggggcc tgaagacctg	720
25	gccaatctga cctcccttcg agtacttgat gtgggtggg attgccgtcg ctgcgaccat	780
	gcccccaatc cctgtataga atgtggccaa aagtccctcc acctgcaccc tgagaccttc	840
	catcacctga gccatctgga aggccctggc ctgaaggaca gctctctcca tacactgaac	900
	tcttcctggc tccaagggtct ggtcaacctc tcgggtctgg acctaagcga gaactttctc	960
	tatgaaagca tcaaccacac caatgcctt cagaaccaa cccgcctgcg caagctcaac	1020
30	ctgtccttca attaccgcaa gaaggatgcc tttggccgccc tccacctggc aagttccttc	1080
	aagaacctgg tgtcaactgca ggagctgaac atgaacggca tcttcttcg ctcgctcaac	1140
	aagtacacgc tcagatggct ggccgatctg cccaaactcc acactctgca tcttcaaattg	1200
	aacttcatca accaggcaca gctcagcatc tttggtacct tccgagccct tcgctttgt	1260
	gacttgcgtac acaatcgcat cagtggccct tcaacgctgt cagaagccac ccctgaagag	1320
35	gcagatgtg cagagcagga ggagctgttg tctgcggatc ctcacccagc tccactgagc	1380
	acccctgttt ctaagaactt catggacagg tgtaagaact tcaagttcac catggacctg	1440
	tctcggaaaca acctgggtgac tatcaagcca gagatgttg tcaatctctc acgcctccag	1500
	tgtcttagcc tgagccacaa ctccattgca caggctgtca atggctctca gttcctgccc	1560
	ctgactaattc tgcaagggtct ggacctgtcc cataacaaac tggacttgta ccactgaaa	1620
40	tcgttcagt agctaccaca gttgcaggcc ctggacctga gctacaacag ccagccctt	1680
	agcatgaagg gtataggcca caatttcagt tttgtggccc atctgtccat gctacacacgc	1740

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	cttagcctgg cacacaatga cattcataacc cgtgtgtcct cacatctcaa cagcaactca	1800
	gtgagggttc ttgacttcag cggcaacggt atgggccgca tgtggatga ggggggcctt	1860
	tatctccatt tcttccaagg cctgagtgcc ctgctgaagc tggacctgtc tcaaaaataac	1920
5	ctgcatatcc tccggccccca gaaccttgac aacctccccca agagcctgaa gctgctgagc	1980
	ctccgagaca actacctatac tttcttaac tggaccagtc tgtccttcct gcccaacctg	2040
	gaagtcctag acctggcagg caaccagcta aaggccctga ccaatggcac cctgccta	2100
	ggcaccctcc tccagaaact ggatgtcagc agcaacagta tcgtctctgt ggtcccagcc	2160
	ttcttcgctc tggcggtcga gctgaaaagag gtcaacctca gccacaacat tctcaagacg	2220
	gtggatcgct cctggtttgg gcccattgtg atgaacctga cagttctaga cgtgagaagc	2280
10	aaccctctgc actgtgcctg tggggcagcc ttcgttagact tactgttggc ggtgcagacc	2340
	aagggtgcctg gcctggctaa tggtgtgaag tgtggcagcc cggccagct gcagggccgt	2400
	agcatcttcg cacaggacct gggcgtgtc ctggatgagg tcctctcttg ggactgctt	2460
	ggccttcac tcttggctgt ggccgtgggc atggtggtgc ctatactgca ccatactctgc	2520
	ggctgggacg tctggtactg ttttcatctg tgcctggcat ggctacctt gctggccgc	2580
15	agccgacgca ggcggcaagg tctcccttat gatgccttcg tggtgttcga taaggcacag	2640
	agcgcagttg cgactgggt gtataacgag ctgcgggtgc ggctggagga gcggcgcgg	2700
	cgccgagccc tacgcttgc tctggaggac cgagattggc tgcctggcca gacgctcttc	2760
	gagaacacctt gggcttccat ctatgggagc cgcaagactc tatttgcgt ggccccacacg	2820
	gaccgcgtca gtggcctcct ggcaccaggc ttccctgctgg ctcagcagcg cctgttggaa	2880
20	gaccgcgaagg acgtgggtgtt gttgggtgatc ctgcgtccgg atgcccacccg ctcccgctat	2940
	gtgcgactgc gccagcgtct ctgcggccag agtgcgtct tctggccca gcagcccaac	3000
	gggcaggggg gcttctgggc ccagctgagt acagccctga cttagggacaa cggccacttc	3060
	tataaccaga acttctgccc gggacactaca gcagaa	3096

25        The deduced amino acid sequence for murine TLR9 (SEQ ID NO:3), comprising 1032 amino acid residues, is shown in Table 4 below in the aligned sequence comparison as mtlr932e.pep. The deduced amino acid sequence for human TLR9 (SEQ ID NO:6), comprising 1032 amino acid residues, is shown in Table 4 below in the aligned sequence comparison as htlr9.pro.

30

**Table 4. Amino Acid Sequence of Murine and Human TLR9**

htlr9.pro	MGFCRSALHPLSLLVQAIMLAMTLALGTLPAFLPCELQPHGLVNCNWLFKSVPHFSMAA	60
mtlr932e.pep	MVLRRRTLHPLSLLVQAAVLAETIALGTLPAFLPCELKPHGLVDCNWLFKSVPRFSAAA	60
35		
htlr9.pro	PRGNVTSLSLSSNRRIHHHLHSDFAHLPNSLRHLNLKWNCPPVGLSPMHFPCHMTIEPSTFL	120
mtlr932e.pep	SCSNITRLSLISNRRIHHHLHNSDFVHLSNLRQLNLKWNCPPTGLSPLHFSCHMTIEPRTFL	120
40		

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	htlr9.pro	AVPTLEELNLSYNNIMTVPALPKSLISLSSLHTNILMDSASLAGLHALRFLFMDGNCYY	180
	mtlr932e.pep	AMRTLEELNLSYNGITTVPRLPSSLVNLSLSHTNIVLDANSLAGLYSLRVLFMDGNCYY	180
			240
5	htlr9.pro	KNPCRQALEVAPGALLGLGNLTHLSLKYNNTVVPRNLPPSLEYLLSYNRTVKLAPEDL	240
	mtlr932e.pep	KNPCTGAVKVTGPAGLLGLSNLTHLSLKYNNTKVRQLPPSLEYLLSYNLTIVKLGPEDL	240
			300
10	htlr9.pro	ANLTALRVLDVGNCRRCDHAPNPCMCPCRHFPQLHPDFTSHSRLEGVLKDSSLWLN	300
	mtlr932e.pep	ANLTSLRVLDVGNCRRCDHAPNPCIECGQKSLHLHPETFHLSHLEGVLKDSSLHTLN	300
			360
	aa197442.pep	LNLNFNYRKVSFARLHLASSF	22
	htlr9.pro	ASWFRLGLNRVLDLSENFLYKCITKTAKAFQGLTQLRKLNLNFNYQKRVSAFAHLSLAPSF	360
15	mtlr932e.pep	SSWFQGLVNLSVLDLSENFLYESINHTNAFQNLTRLRKLNLNFNYRKVSFARLHLASSF	360
			420
	mousepep1	C	1
	aa197442.pep	KNLVSLQELNMNGIFFRLLNKYTLRWLADLPKLHTLHQMNFINQAQLSIFGTFRALRFV	82
20	htlr9.pro	GSLVALKELEDMHGIFFRSLETTLRPLARLPMLQTRLQMNFINQAQLGIIFRAFPGLRYV	420
	mtlr932e.pep	KNLVSLQELNMNGIFFRSLNKTYTLRWLADLPKLHTLHQMNFINQAQLSIFGTFRALRFV	420
			480
	mousepep1	DLSDRNRISGPSTLSEA	17
25	humanpep1	PAPVDTPSSEDFRPNC	16
	aa197442.pep	DLSDRNRISGPSTLSEATPEEADDAEQEELLSADPHPAPLSTPASKNFMDRCKNFKFNMDL	142
	htlr9.pro	DLSDRNRISGASELT-ATMGEADGGEKVWLQPGDLAPAPVDTPSSEDFRPNCSTLNFTLDL	479
	mtlr932e.pep	DLSDRNRISGPSTLSEATPEEADDAEQEELLSADPHPAPLSTPASKNFMDRCKNFKFTMDL	480
30			540
	aa197442.pep	SRNNLVTTAEMFVNLSRLQCLSLSHNSIAQAVNGS	178
	htlr9.pro	SRNNLVTVQPEMFVNLQCLRLSHNCISQAVNGSQFLPLTGLQVLDLSRNKLDLYHEH	539
	mtlr932e.pep	SRNNLVTIKPEMFVNLSRLQCLSLSHNSIAQAVNGSQFLPLTNLQVLDLSHNKLDLYHWK	540
35			600
	aa162495.pep	YNSQPFMSMKIGIGHNFSFVTHLSMLQSLSLAHNDIHTRVSSHLSNS	46
	htlr9.pro	SFTELPRLEALDLSYNSQPFGMQGVGHNFVFVAHLRTLRLSLAHNNIHQSQQQLCSTS	599
	mtlr932e.pep	SFSELPOQLQALDLSYNSQPFMSMKIGIGHNFSFVAHLSMLHSLSLAHNDIHTRVSSHLSNS	600
40			660
	aa162495.pep	VRFLDFSGNGMGRMWDEGGYLHFFQGLSGVLKLDLSQLNNLHILRPQNLDNLPKSLKLLS	106
	htlr9.pro	LRALDFSGNALGHMWAEGDLYLHFFQGLSGLIWLDLSQLNRLHTLLPQTLRNLPKSLQVLR	659
	mtlr932e.pep	VRFLDFSGNGMGRMWDEGGYLHFFQGLSGLLKLDLSQLNNLHILRPQNLDNLPKSLKLLS	660
45			720
	aa162495.pep	LRDNYLSFFNWTSLSFLPNLEVLDLAGNQLKALTNGTLPNGTLLQKLDVSSNSIVS	162

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	htlr9.pro	LRDNYLAFFKWWSLHFLPKLEVLDLAGNRLKALTNGSLPAGTRLRRLDVSCNSISFVAPG	719
	mtlr932e.pep	LRDNYLSFFNWTSLSFLPNLEVLDLAGNQLKALTNGTLLQKLDVSSNSIVSVVPA	720
			780
5	ai451215.pep	PIVMNLTVLDVRSNPLHCACGAAFDLLEVQT	33
	htlr9.pro	FFSKAKELRELNLSANALKTVDHSGWFGPLASALQILDVSANPLHCACGAAFMDFLLEVQA	779
	mtlr932e.pep	FFALAVELKEVNLSHNLKTVDRSWFGPIVMNLTVLDVRSNPLHCACGAAFDLLEVQT	780
			840
10	ai451215.pep	KVPGLANGVKCGSPGQLQGRSIFAQDLRLCLDEVLSWDCFGSLLAVAVGMVVPILHHLC	93
	htlr9.pro	AVPGLPSRVKCGSPGQLQGLSIFAQDLRLCLDEALSWDCFALSLLAVALGLGVPMILHHLC	839
	mtlr932e.pep	KVPGLANGVKCGSPGQLQGRSIFAQDLRLCLDEVLSWDCFGSLLAVAVGMVVPILHHLC	840
			900
15	ai451215.pep	GWDVWYCFHLCLAWLPILLAR-SRRSAQTLPYDAVVFDKAQSAVADWVYNELRVLEERR	152
	htlr9.pro	GWDLWYCFHLCLAWLPWRGRQSGRDEDALPYDAVVFDKTQSAVADWVYNELRGQLEECR	899
	mtlr932e.pep	GWDVWYCFHLCLAWLPILLAR-SRRSAQALPYDAVVFDKAQSAVADWVYNELRVLEERR	899
			960
20	aa273731.pep	AHTDRVSGLLRTSFLLAQQRLL	22
	ai463056.pep	EDRDWLPGQTLFENLWASIYGSRKTLFVLAHTDRVSGLLRTSFLLAQQRLL	51
	ai451215.pep GR		154
	htlr9.pro	GRWALRLCLEERDWLPGKTLFENLWASVYGSRKTLFVLAHTDRVSGLLRASFLLAQQRLL	959
	mtlr932e.pep	GRRALRLCLEDRWLPGQTLFENLWASIYGSRKTLFVLAHTDRVSGLLRTSFLLAQQRLL	959
25			
	humanpep2	H	1
	mousepep2	H	1
	aa273731.pep	EDRKDVVVLVILRPDAXPSRYVRLRQRLCRQSVLFWPQRPNGQGGFWAQLSTALTRDNRH	82
30	ai463056.pep	EDRKDVVVLVILRPDAHRSRYVRLRQRLCRQSVLFWPQQPNGQGGFWAQLSTALTRDNRH	111
	htlr9.pro	EDRKDVVVLVILSPDGRRSRYVRLRQRLCRQSVLLWPHQPSGQRSFWAQLGMALTRDNHH	1019
	mtlr932e.pep	EDRKDVVVLVILRPDAHRSRYVRLRQRLCRQSVLFWPQQPNGQGGFWAQLSTALTRDNRH	1019
			1080
35	humanpep2	FYNRNFCQGPTAE	14
	mousepep2	FYNQNFCRGPTAE	14
	aa273731.pep	FYNQNFCRGPTAE	95
	ai463056.pep	FYNQNFCRGPTA	123
	htlr9.pro	FYNRNFCQGPTAE	1032
40	mtlr932e.pep	FYNQNFCRGPTAE	1032

The following SEQ ID NOs correspond to the sequences as shown in Table 4:

htlr9.pro: SEQ ID NO:6; mtlr932e.pep: SEQ ID NO:3; aa197442.pep: SEQ ID NO:8;  
 mousepep1: SEQ ID NO:17; humanpep1: SEQ ID NO:19; aa162495.pep: SEQ ID NO:14;

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ai451215.pep: SEQ ID NO:16; aa273731.pep: SEQ ID NO:10; ai463056.pep: SEQ ID NO:12; humanpep2: SEQ ID NO:20; and mousepep2: SEQ ID NO:18.

**Example 2. Reconstitution of TLR9 signaling in 293 fibroblasts**

5        The cloned mouse TLR9 cDNA (see above) and human TLR9 cDNA (gift from B. Beutler, Howard Hughes Medical Institute, Dallas, TX) in pT-Adv vector (from Clonetech) were cloned into the expression vector pcDNA3.1(-) from Invitrogen using the EcoRI site. Utilizing a “gain of function” assay it was possible to reconstitute human TLR9 (hTLR9) and murine TLR9 (mTLR9) signaling in CpG DNA non-responsive human 293 fibroblasts  
10      (ATCC, CRL-1573). The expression vectors mentioned above were transfected into 293 fibroblast cells using the calcium phosphate method.

Since NF- $\kappa$ B activation is central to the IL-1/TLR signal transduction pathway (Medzhitov R et al., *Mol Cell* 2:253-258 (1998); Muzio M et al., *J Exp Med* 187:2097-2101 (1998)), cells were transfected with hTLR9 or co-transfected with hTLR9 and a NF- $\kappa$ B-15 driven luciferase reporter construct. Human fibroblast 293 cells were transiently transfected with (**Figure 1A**) hTLR9 and a six-times NF- $\kappa$ B-luciferase reporter plasmid (NF- $\kappa$ B-luc, kindly provided by Patrick Baeuerle, Munich, Germany) or (**Figure 1B**) with hTLR9 alone. After stimulus with CpG-ODN (2006, 2 $\mu$ M, TCGTCGTTTGTCGTTTGTCGTT, SEQ ID NO:112), GpC-ODN (2006-GC, 2 $\mu$ M, TGCTGCTTTGTGCTTTGTGCTT, SEQ ID NO:118), LPS (100 ng/ml) or media, NF- $\kappa$ B activation by luciferase readout (8h, **Figure 1A**) 20 or IL-8 production by ELISA (48h, **Figure 1B**) were monitored. Results are representative of three independent experiments. **Figure 1** shows that cells expressing hTLR9 responded to CpG-DNA but not to LPS.

**Figure 2** demonstrates the same principle for the transfection of mTLR9. Human 25 fibroblast 293 cells were transiently transfected with mTLR9 and the NF- $\kappa$ B-luc construct (**Figure 2**). Similar data was obtained for IL-8 production (not shown). Thus expression of TLR9 (human or mouse) in 293 cells results in a gain of function for CpG-DNA stimulation similar to hTLR4 reconstitution of LPS responses.

To generate stable clones expressing human TLR9, murine TLR9, or either TLR9 30 with the NF- $\kappa$ B-luc reporter plasmid, 293 cells were transfected in 10 cm plates (2x10<sup>6</sup> cells/plate) with 16  $\mu$ g of DNA and selected with 0.7 mg/ml G418 (PAA Laboratories GmbH,

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Cölbe, Germany). Clones were tested for TLR9 expression by RT-PCR, for example as shown in **Figure 3**. The clones were also screened for IL-8 production or NF- $\kappa$ B-luciferase activity after stimulation with ODN. Four different types of clones were generated.

- 5      293-hTLR9-luc:            expressing human TLR9 and 6-fold NF- $\kappa$ B-luciferase reporter  
293-mTLR9-luc:            expressing murine TLR9 and 6-fold NF- $\kappa$ B-luciferase reporter  
293-hTLR9:                expressing human TLR9  
293-mTLR9:                expressing murine TLR9

10       **Figure 4** demonstrates the responsiveness of a stable 293-hTLR9-luc clone after stimulation with CpG-ODN (2006, 2 $\mu$ M), GpC-ODN (2006-GC, 2 $\mu$ M), Me-CpG-ODN (2006 methylated, 2 $\mu$ M; TZGTZGTTTGTZGTTTGTT, Z = 5-methylcytidine, SEQ ID NO:128), LPS (100 ng/ml) or media, as measured by monitoring NF- $\kappa$ B activation. Similar results were obtained utilizing IL-8 production with the stable clone 293-hTLR9.  
15       293-mTLR9-luc were also stimulated with CpG-ODN (1668, 2 $\mu$ M; TCCATGACGTTCCCTGATGCT, SEQ ID NO:84), GpC-ODN (1668-GC, 2 $\mu$ M; TCCATGAGCTTCCTGATGCT, SEQ ID NO:85), Me-CpG-ODN (1668 methylated, 2 $\mu$ M; TCCATGAZGTTCCCTGATGCT, Z = 5-methylcytidine, SEQ ID NO:207), LPS (100 ng/ml) or media, as measured by monitoring NF- $\kappa$ B activation (**Figure 5**). Similar results were  
20       obtained utilizing IL-8 production with the stable clone 293-mTLR9. Results are representative of at least two independent experiments. These results demonstrate that CpG-DNA non-responsive cell lines can be stably genetically complemented with TLR9 to become responsive to CpG DNA in a motif-specific manner. These cells can be used for screening of optimal ligands for innate immune responses driven by TLR9 in multiple species.

25

**Example 3. Expression of soluble recombinant human TLR9 in yeast cells (*Pichia pastoris*)**

Human TLR9 cDNA coding for amino acids 1 to 811 was amplified by PCR using the primers 5'-ATAGAATTCAATAATGGGTTCTGCCGCAGCGCCCT-3' (SEQ ID NO:194) and 5'-ATATCTAGATCCAGGCAGAGGCGCAGGTC-3' (SEQ ID NO:195), digested with EcoRI and XbaI, cloned into the yeast expression vector pPICZB (Invitrogen, Groningen,

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Netherlands) and transfected into yeast cells (*Pichia pastoris*). Clones were selected with the antibiotic zeozin and protein production of soluble human TLR9 was induced with methanol (see **Figure 6**: SDS-PAGE, Coomassie stained, arrow marks hTLR9; lane 1: supernatant of culture induced with methanol; lane 2: supernatant of culture not induced). Thus TLR9  
5 protein can be isolated from transfectants and further utilized for protein studies and vaccination purposes.

**Example 4. hTLR9 expression correlates with CpG-DNA responsiveness.**

Bacterial DNA has been described as a mitogen for both murine and human B cells.

10 Although LPS is also mitogenic for murine B cells, it is generally accepted that LPS is not a mitogen for human B cells. **Figure 7** demonstrates that human B cells proliferate after stimulation with *E. coli* DNA or a CpG-ODN but not Dnase-digested *E. coli* DNA or a control GpC-ODN. Purified human B cells were stimulated with 50 $\mu$ g/ml *E. coli* DNA, a comparable amount of DNase I-digested *E. coli* DNA, 2 $\mu$ M CpG-ODN (2006), 2 $\mu$ M GpC-  
15 ODN (2006-GC) or 100 ng/ml LPS. B cell proliferation was monitored at day two by  $^3$ H-thymidine uptake. These data demonstrate that it was DNA within the *E. coli* DNA preparation that was mitogenic and that a CpG-motif within the ODN was required.

Human dendritic cells (DC) have been claimed to be responsive to CpG-DNA. While analyzing human dendritic cell responses to CpG-DNA, we noted that plasmacytoid DC  
20 (CD123+DC) produced IFN- $\alpha$ , TNF, GM-CSF, and IL-8 upon exposure to CpG-DNA but not to LPS (**Figure 8** and unpublished data). The converse was true for stimulation of monocyte-derived dendritic cells (MDDC) (**Figure 8** and unpublished data). Purified CD123+DC or MDDC were stimulated with 50 $\mu$ g/ml *E. coli* DNA, a comparable amount of DNase I-digested *E. coli* DNA, 2 $\mu$ M CpG-ODN (2006), 2 $\mu$ M GpC-ODN (2006-GC) or 100 ng/ml LPS (**Figure 8**). IL-8 and TNF concentration was determined by enzyme-linked immunosorbent assay (ELISA). The CD123+DC response was DNA- and CpG-motif restricted. Monocyte-derived dendritic cells (MDDC) however demonstrated the converse response pattern, a response to LPS but not CpG-DNA. Due to this segregated response we analyzed TLR expression.  
25

30 We have shown that CpG-DNA utilizes the Toll/IL-1R (TIR) signal transduction pathway implying the need for a TIR domain in the CpG-DNA signaling receptor. Häcker H

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et al., *J Exp Med* 192:595-600 (2000). It was further demonstrated that TLR9-deficient mice are non-responsive to CpG-ODN. Hemmi H et al., *Nature* 408:740-5. By semi-quantitative RT-PCR both B cells and CD123+ DC yielded positive signals for hTLR9 while MDDC, monocytes and T cells were weak to negative (**Figure 9**). The cDNAs were prepared from 5 monocyte-derived dendritic cells (MDDC), lane 1; purified CD14+ monocytes, lane 2; B cells, lane 3; CD123+ DC, lane 4; CD4+ T cells, lane 5; and CD8+ T cells, lane 6. cDNA amounts were normalized based on GAPDH amount determined by TAG-MAN PCR (Perkin-Elmer). RT-PCR was performed for 30 cycles on normalized cDNA diluted 1:5 for human 10 TLR2, 4 and 9, while GAPDH was diluted 1:125. We also tested for hTLR2 and hTLR4 expression. MDDC and monocytes were positive while B cells, T cells and CD123+DC were weak to negative (**Figure 9**). Weak signals delivered by PCR could be explained by contaminating cells, however a strong positive signal implies expression. These data 15 demonstrated a clear correlation between hTLR9 mRNA expression and B cell or CD123+DC responsiveness to CpG-DNA (**Figures 7 and 8**). A correlation could also be shown for hTLR2 and hTLR4 expression and MDDC responsiveness to LPS (**Figure 8**). This data demonstrates that hTLR9 is a relevant receptor for CpG-DNA responses and that its expression determines responsiveness. If TLR9 expression could be modulated, agonism or antagonism of CpG-DNA responses could be achieved.

20 **Example 5. Species specificity of TLR9 signaling**

By iterative examination of the flanking sequences surrounding CG dinucleotides, CpG-motifs have been identified. Paradoxically, or by twist of nature, the human optimal CpG-motif, GTCGTT (SEQ ID NO:66), is different from the murine motif, GACGTT (SEQ ID NO:129). Human peripheral blood mononuclear cells (PBMC) (**Figure 10A**) and murine 25 splenocytes (**Figure 10B**) were stimulated with ODN 2006 (filled circle, TCGTCGTTTGTCTTTGTCGTT, SEQ ID NO:112), ODN 2006-GC (open circle), ODN 1668 (filled triangle, TCCATGACGTTCCTGATGCT, SEQ ID NO:84) or ODN 1668-GC (open triangle, TCCATGAGGCTCCTGATGCT, SEQ ID NO:85) at indicated concentrations and IL-12 production was monitored after 8 hours. **Figure 10A** shows that titration of the 30 optimal human ODN, 2006, on PBMC induces IL-12 production. The optimal murine sequence, 1668, however was much less effective in eliciting IL-12 from PBMC. The two

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control GpC-ODNs were essentially negative. The converse was true for murine splenocytes (Figure 10B), in that the murine sequence induced optimal IL-12 while the human sequence was much less effective. It should also be noted that the  $K_{ac}$  (concentration of half-maximal activation) of murine splenocytes for 1668 was greater than human PBMC for 2006 (compare 5 **Fig. 10A to Fig. 10B**).

Because stable TLR9 transfectants mirrored primary cell responsiveness to CpG-DNA (Figures 4 and 5), it was hypothesized that stable transfectants could potentially discern species-specific CpG-motifs through TLR9 receptors. Therefore 293-hTLR9-luc (expressing human TLR9 and 6-fold NF- $\kappa$ B-luc reporter), 293-mTLR9-luc (expressing murine TLR9 and 10 6-fold NF- $\kappa$ B-luc reporter), 293-hTLR9 (expressing human TLR9) and 293-mTLR9 (expressing murine TLR9) clones were tested for CpG-DNA motif responsiveness. Figure 11 shows titration curves for 2006 or 1668 and their controls versus either hTLR9 or mTLR9 15 cells. Depicted are both NF- $\kappa$ B-driven luciferase and IL-8 production as readout. In both 293 hTLR9-luc and 293-mTLR9-luc cells stimulation with CpG-DNA resulted in NF- $\kappa$ B activation, as determined by measurement of the induced expression of firefly luciferase under the control of a minimal promotor containing six tandem NF- $\kappa$ B-binding sites. After lysis of the cells luciferase can be detected photometrically based on an enzymatic reaction by luciferase which creates photons. IL-8 production was monitored using enzyme-linked immunosorbent assay (ELISA). Figure 11 depicts clones stimulated with ODN 2006 (filled 20 circle), ODN 2006-GC (open circle), ODN 1668 (filled triangle) or ODN 1668-GC (open triangle) at indicated concentrations and NF- $\kappa$ B activation or IL-8 production were measured after 10 and 48 hours, respectively. Results shown in Figure 11 are representative of three independent experiments. Strikingly, CpG-motif sequence specificity was conferred in a species-specific manner by TLR9. Additionally, the half-maximal concentration for either 25 2006 or 1668 appears nearly the same as those determined on primary cells (compare Figure 10 and Figure 11). These data demonstrate that TLR9 is the CpG-DNA receptor and that exquisite specificity to CpG-DNA sequence is conferred by TLR9.

**Example 6. Use of stable TLR9 clones to test responsiveness to substances other than 30 phosphorothioate ODN**

As described in the foregoing Examples, the stable TLR9 clones were initially

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screened for fidelity of phosphorothioate CpG-ODN reactivity. The 293-hTLR9 cells demonstrated reactivity to CpG-DNA and not LPS in a CpG-motif dependent manner (**Figures 4 and 5**). In the present example the stable TLR9 transfectants were tested for responsiveness to additional DNAs. NF- $\kappa$ B activation was monitored after stimulation with 5 *E. coli* DNA (black bars) or *E. coli* DNA digested with DNase I (gray bars) in 293-hTLR9-luc cells. **Figure 12** demonstrates an *E. coli* DNA dose-dependent induction of NF- $\kappa$ B-driven luciferase expression to a level comparable to phosphorothioate CpG-ODN (**Figure 11**). Activity was destroyed by DNase I digestion, indicating specificity of response to DNA and not contaminant bacterial products. The stable TLR9 transfectants can be used to screen the 10 activity of DNAs from various species or vector DNAs intended for immune system stimulation. In particular, TLR9 transfectants can be used to screen and compare the immunostimulatory activity of DNAs from various species of pathogens, DNA constructs, DNAs intended for use as vaccines, gene replacement therapeutics, and nucleic acid vectors.

293-hTLR9-luc cells also were stimulated with the phosphodiester variants of ODN 15 2006 (filled circle), ODN 2006-GC (open circle), ODN 1668 (filled triangle) or ODN 1668-GC (open triangle) at indicated concentrations, and NF- $\kappa$ B activation was monitored after 12 hours (**Figure 13A**). Likewise, 293-mTLR9-luc cells were stimulated with the phosphodiester variants of ODN 2006 (filled circle), ODN 2006-GC (open circle), ODN 1668 (filled triangle) or ODN 1668-GC (open triangle) at indicated concentrations, and NF- $\kappa$ B 20 activation was monitored after 12 hours (**Figure 13B**). These assays show that the stable TLR9 transfectants responded to DNAs other than phosphorothioate-modified ODN. These data demonstrate the utility of stable TLR transfectants for screening for agonists of the TLR9 receptor.

25 **Example 7. TLR9 determines CpG-ODN activity**

Although 2006 and 1668 are discussed in terms of CpG-motif differences, they are very different in several aspects (see Table 5 for comparison). The lengths are different, 24 versus 20 nucleotides, and 2006 has four CG dinucleotides compared to one in 1668. Additional differences are the CG position relative to the 5' and 3' ends and also 5' sequence 30 differences. In order to determine if motif specificity is a quality of the motif and not the global sequence environment, for this experiment several sequences were produced holding

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these variables constant. As a starting point, the 1668 sequence was modified by converting the central C to T and the distal TG to CG, thereby creating a second CG in the resulting sequence 5000 (SEQ ID NO:130, Table 5). Then point nucleotide changes were made, progressing toward a 2006-like sequence, 5007 (SEQ ID NO:98). The ODN 5002 (SEQ ID

5 NO:132) is most like 1668 with the exception that C's at positions 12 and 19 have been converted to T's. The last 16 nucleotides of ODN 5007 are the same as the last15 nucleotides of 2006 with the exception of an additional T. The ODN concentration of half-maximal activation ( $K_{ac}$ ) was determined by producing ODN titration curves using either 293-hTLR9-luc or 293-mTLR9-luc cells and NF- $\kappa$ B-driven luciferase expression as a readout. Example 10 curves are given in **Figure 14**. Stable transfectants 293-hTLR9-luc and 293-mTLR9-luc were stimulated with ODN 5002 (filled circle) or ODN 5007 (open circle) at indicated concentrations and NF- $\kappa$ B activation was monitored after 12 hours. Results shown in **Figure 14** are representative of three independent experiments. Values for  $K_{ac}$  for multiple ODN are given in Table 5. Similar results were obtained for those ODN tested with 293-hTLR9 and 15 293-mTLR9 cells utilizing IL-8 as readout.

**Table 5. CpG-DNA sequence specificity of human and murine TLR9 signaling activity**

CpG-DNA	Sequence	293-hTLR9	293-mTLR9	SEQ ID NO:
		$K_{ac}$ (nM)	$K_{ac}$ (nM)	
1668	TCCAT <u>GACGTT</u> CCTGATGCT	>10,000	70	84
1668-GC	TCCAT <u>GAGCTT</u> CCTGATGCT	>10,000	>10,000	85
2006	TC <u>GTCGTTTGTCGTTTGTCGTT</u>	400	>10,000	112
2006-GC	TGCT <u>GCTTTGTGCTTTGTGCTT</u>	>10,000	>10,000	118
5000	TCCAT <u>GACGTT</u> CTTGACGCT	10,000	82	130
5001	TCCATGACGTTCTTGACGTT	7,000	55	131
5002	TCCATGACGTTCTTGATGTT	7,000	30	132
5003	TCCATGACGTTTTGATGTT	10,000	30	133
5004	TCCATGTCGTTCTGATGTT	5,000	400	134
5005	TCCATGTCGTTTTGATGTT	3,000	2,000	135
5006	TCCATGTCGTTTTGTTGTT	3,000	650	136
5007	TCCAT <u>GTCGTTTGTCGTT</u>	700	1,000	98
5002	TCCAT <u>GACGTT</u> CTTGATGTT	ND	30	132
5008	TCCATGACGTTATTGATGTT	ND	40	137
5009	TCCATGACGTCCTTGATGTT	ND	>10,000	138
5010	TCCATGACGT <u>CATTGATGTT</u>	ND	>10,000	139

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In previous unpublished work by the inventors, it had been noted that a CA substitution converting the mouse CpG-motif from GACGTTC to GACGTCA was deleterious. To extend our examination of the motif, three more ODN were created to dissect this effect (5008-5010, SEQ ID NOs:137-139, Table 5).

The activity displayed by the 293-hTLR9-luc clone increased with progressive nucleotide substitutions converting the mouse sequence toward the human sequence (Table 5, sequences 5000-5007). The converse was true for the 293-mTLR9-luc clone, which showed highest activity for the mouse sequences. The originally hypothesized CpG-motif was purine-purine-CG-pyrimidine-pyrimidine. Most notable to motif definition as determined by TLR9 genetic complementation was the non-conservative pyrimidine for purine change A to T immediately 5' of the CG (Table 5). These changes improved 293-hTLR9-luc responsiveness but diminished 293-mTLR9-luc responsiveness. These results support the notion that the preferred mouse motif contains ACG while the preferred human sequence contains TCG. The conservative pyrimidine for pyrimidine change T to C in the mouse motif, ACGTT versus ACGTC (5002 versus 5009), completely destroyed 293-mTLR9 responsiveness. Although not a complete iterative analysis of the CpG-motif, the data refine our understanding of the motif. More importantly these data strongly support direct CpG-motif engagement by TLR9.

## 20 Example 8. Antagonist definition

It has been demonstrated that DNA uptake and endosomal maturation are required for signal initiation by CpG-DNA. It has been hypothesized that in order for DNA to enter the endosomal/lysosomal compartment a non-CpG dependent uptake receptor may be required. 293 cells were transiently transfected with mTLR9 treated with either medium only or 1.0  $\mu$ M 25 CpG-ODN 1668 (Figure 15). Additionally the 1668-treated TLR9 transfectants were simultaneously exposed to various doses of a non-CpG ODN (PZ2; 5'-CTCCTAGTGGGGGTGTCCTAT-3', SEQ ID NO:43). IL-8 production was monitored after 48h by ELISA. Figure 15 shows that PZ2, in a dose-dependent manner, was able to antagonize the activation of TLR9-transfected cells stimulated with a CpG ODN.

30 **Figure 16** demonstrates that the stable TLR9 transfectants, 293-hTLR9-luc cells, are sensitive to non-CpG-ODN blockade. 293-hTLR9-luc cells were incubated with CpG-ODN

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(0.5  $\mu$ M) (black bars) or TNF- $\alpha$  (10 ng/ml) (gray bars) and increasing concentrations of a blocking ODN (5'-HHHHHHHHHHHHHHWGGGGG-3', SEQ ID NO:140; H = A, T, C; W = A, T) as indicated. NF- $\kappa$ B activation was monitored after 12 hours and is presented as percent yields. Thus both mTLR9 and hTLR9 activity can be blocked by non-stimulatory  
5 ODN. The blockade is specific to blocking ODN since the TNF-driven NF- $\kappa$ B signal was not diminished. Antagonism of CpG-DNA responses could thus be defined in stable TLR9 cells and therefore high throughput screening can be done for TLR9 antagonist.

Bafilomycin A poisons the proton pump needed for H<sup>+</sup> transport into endosomes, which is required for endosomal maturation. **Figure 17** shows that blockade of endosomal 10 maturation in 293-hTLR9-luc cells fully blocks CpG-ODN induction of NF- $\kappa$ B. 293-hTLR9-luc cells were preincubated with 10 nM Bafilomycin A (gray bars) or dimethylsulfoxide (DMSO) control (black bars) for 30 min and stimulated with CpG-ODN (2006, 0.5  $\mu$ M), IL-1 (10 ng/ml) or TNF- $\alpha$  (10 ng/ml) as indicated. NF- $\kappa$ B activation was monitored after 12 hours and is presented as percent yields. The blockade was specific to CpG-DNA generated signal  
15 because both IL-1 and TNF induction of NF- $\kappa$ B was unaffected. These data demonstrate that 293 cells stably complemented with hTLR9 behave in a manner similar to primary CpG-DNA responsive cells, in that cellular uptake and endosomal maturation are required for induction of signal by CpG-DNA. Thus the stable transfectants can be used as indicator for TLR9 drug antagonist.

CpG-DNA signaling appears to occur via a Toll/IL-1R-like pathway. It was shown in 20 the mouse that CpG-DNA signaling is dependent on MyD88, IRAK and TRAF6. Häcker H et al., *J Exp Med* 192:595-600 (2000). Hemmi et al. demonstrated that mTLR9-deficient mice lack activation of IRAK upon CpG-ODN stimulation. Hemmi H et al., *Nature* 408:740-5 (2000). **Figure 18** shows that CpG-DNA signaling via human TLR9 was MyD88  
25 dependent. hTLR9 (293-hTLR9) was co-transfected with a six-times NF- $\kappa$ B luciferase reporter plasmid and increasing concentrations of the dominant negative human MyD88 expression vector. Cells were not stimulated (filled circles), stimulated with CpG-ODN (2006, 2 $\mu$ M) (open circles) or TNF- $\alpha$  (10 ng/ml) (filled triangles) and NF- $\kappa$ B activation was monitored after 12 hours. Results are representative of at least two independent experiments.  
30 **Figure 18** demonstrates that dominant negative MyD88 blocks NF- $\kappa$ B induction in 293-hTLR9 cells following CpG-DNA stimulation. The blockade of MyD88 did not affect NF-

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κB induction via TNF induced signal transduction. In general these data confirm the central role of MyD88 to TLR signaling and specifically the role of MyD88 in CpG-DNA initiation of signal. Thus human cells transfected with TLR9 can be used as indicators to find molecules to antagonize CpG-DNA via genetic mechanisms.

5

#### **Example 9. Antibody production**

Peptides for human and mouse TLR9 were designed for coupling to a carrier protein and injected into rabbits to obtain anti-peptide polyclonal antisera. Mouse peptide 1 (mousepep1, see Table 4) can be found in EST aa197442 and peptide 2 (mousepep2, see Table 4) in EST aa273731 and ai463056. Human peptide 1 (humanpep1, see Table 4) and peptide 2 (humanpep2, see Table 4) were taken from the published human sequence.

Three rabbit antisera were generated by this method: anti-mousepep1, specific for the extracellular domain of murine TLR9; anti-humanpep1, specific for the extracellular domain of hTLR9; and antisera against a combination of mousepep2 and humanpep2, specific for the cytoplasmic domain of both murine and human TLR9. Immunoprecipitates with anti-FLAG antibody were electrophoresed by PAGE and, using standard Western blotting techniques, transferred to membrane and probed with the various antisera. **Figure 19** shows the response to hTLR9-FLAG and mTLR9-FLAG. The TLR9 in these blots are indicated with arrows, while the lower molecular weight bands represent anti-FLAG antibody.

20

#### **Example 10. Mutation adjacent to the CXXC-domain (hTLR9-CXXCm, mTLR9-CXXCh)**

The CXXC motif resembles a zinc finger motif and is found in DNA-binding proteins and in certain specific CpG binding proteins, e.g. methyl-CpG binding protein-1 (MBD-1).

25 Fujita N et al., *Mol Cell Biol* 20:5107-5118 (2000). Human and murine TLR9 contain two CXXC motifs. The CXXC domain is highly conserved between human and murine TLR9 but followed by 6 amino acids (aa) which differ quite substantially in polarity and size. By the use of a site-specific mutagenesis kit (Stratagene, La Jolla, CA, USA) these six amino acid residues (human: PRHFPQ 269-274); mouse: GQKSLH 269-274) were interchanged between 30 human and murine TLR9. These mutations were generated by the use of the primers 5'-CTGCATGGAGTGC GGCCAAAGTCCCTCCACCTACATCCCGATAC-3' (SEQ ID

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NO:141) and

5'-GTATCGGGATGTAGGTGGAGGGACTTTGCCGCACCCATGCAG-3' (SEQ ID NO:142) for human TLR9 and the primers

5'-CTGTATAGAACATGTCCTCGTCACCTCCCCAGCTGCACCCTGAGAC-3' (SEQ ID

5 NO:143) and

5'-GTCTCAGGGTGCAGCTGGGGAAAGTGACGAGGACATTCTATACAG-3' (SEQ ID NO:144) for murine TLR9 according to the manufacturer's protocol.

CXXC motif:	CXXCXXXXXXCXXC	SEQ ID NO:145
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10 Wildtype hTLR9:	CRRCDHAPNPCMECPRFHQ	aa 255-274	SEQ ID NO:146
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hTLR9-CXXCm:	CRRCDHAPNPCME <u>CQKSLH</u>	aa 255-274	SEQ ID NO:147
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Wildtype mTLR9:	CRRCDHAPNPCMIC <u>QKSLH</u>	aa 255-274	SEQ ID NO:148
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mTLR9-CXXCh:	CRRCDHAPNPCMIC <u>PRHFQ</u>	aa 255-274	SEQ ID NO:149
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15 For the stimulation of the hTLR9 variant hTLR9-CXXCm, 293 cells were transiently transfected with hTLR9 or hTLR9-CXXCm and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 20**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. The data show that hTLR9 can be improved by converting the human CXXC domain to the murine CXXC domain. For the stimulation of the mTLR9 variant mTLR9-CXXCh, 293 cells were transiently transfected with mTLR9 or mTLR9-CXXCh and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 21**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. It appears that the human CXXC domain may diminish mTLR9-CXXCh activity relative to the wild type mTLR9.

#### **Example 11. Mutation in the MBD motif (hTLR9-MBDmut, mTLR9-MBDmut)**

The MBD motif is a domain recently described for CpG binding in the protein MBD-

1. Fujita N et al., *Mol Cell Biol* 20:5107-5118 (2000); Ohki I et al., *EMBO J* 18:6653-6661

30 (1999). Human and murine TLR9 contain this motif at position 524-554 and 525-555, respectively.

MBD-1	R-XXXXXXX-R-X-D-X-Y-XXXXXXXXX-R-S-XXXXXX-Y	SEQ ID NO:125
hTLR9	Q-XXXXXXX-K-X-D-X-Y-XXXXXXXXX-R-L-XXXXXX-Y	SEQ ID NO:126
mTLR9	Q-XXXXXXX-K-X-D-X-Y-XXXXXXXXX-Q-L-XXXXXX-Y	SEQ ID NO:127

5

The core of this domain consists of D-L-Y in human TLR9 (aa 534-536) and mouse TLR9 (aa 535-537). Through site-specific mutagenesis D534 and Y536 in human TLR9, and D535 and Y537 in murine TLR9, were mutated to alanines creating the sequence A-L-A for human (aa 534-536) and murine TLR9 (aa 535-537). These mutations were generated by the use of the primers 5'-CACAAATAAGCTGGCCCTGCCACGAGCACTC-3' (SEQ ID NO:150) and 5'-GAGTGCTCGTGGCGAGGCCAGCTTATTGTG-3' (SEQ ID NO:151) for human TLR9 and the primers 5'-CATAACAAACTGGCCTGGCCCAGTGGAAATC-3' (SEQ ID NO:152) and 5'-GATTCCAGTGGCCAAGGCCAGTTGTATG-3' (SEQ ID NO:153) for murine TLR9 according to the manufacturer's protocol.

15 For the stimulation of mTLR9 variant, mTLR9-MBDmut, 293 cells were transiently transfected with mTLR9 or mTLR9-MBD-mut and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 22**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. For the stimulation of hTLR9 variant, hTLR9-MBDmut, 293 cells were transiently transfected with hTLR9 or 20 hTLR9-MBD-mut and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 23**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. The disruption of the putative CpG binding domain DXY in TLR9 destroyed receptor activity. These data demonstrate that the MBD motif is most likely involved in CpG-DNA binding and can be thus be manipulated to better 25 understand CpG-DNA binding and efficacy.

**Example 12. Proline to Histidine mutation in the TIR-domain (hTLR9-PHmut, mTLR9-PHmut)**

Toll-like receptors have a cytoplasmic Toll/IL-1 receptor (TIR) homology domain 30 which initiates signaling after binding of the adapter molecule MyD88. Medzhitov R et al., *Mol Cell* 2:253-8 (1998); Kopp EB et al., *Curr Opin Immunol* 11:15-8 (1999). Reports by

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others have shown that a single-point mutation in the signaling TIR-domain in murine TLR4 (Pro712 to His) or human TLR2 (Pro681 to His) abolishes host immune response to lipopolysaccharide or gram-positive bacteria, respectively. Poltorak A et al., *Science* 282:2085-8 (1998); Underhill DM et al., *Nature* 401:811-5 (1999). Through site-specific 5 mutagenesis the equivalent Proline at position 915 of human and murine TLR9 were mutated to Histidine (Pro915 to His). These mutations were generated by the use of the primers 5'-GCGACTGGCTGCATGGCAAAACCCCTTTG-3' (SEQ ID NO:154) and 5'-CAAAGAGGGTTTGCCATGCAGCCAGTCGC-3' (SEQ ID NO:155) for human TLR9 and the primers 5'-CGAGATTGGCTGCATGGCCAGACGCTCTTC-3' (SEQ ID NO:156) 10 and 5'-GAAGAGCGTCTGCCATGCAGCCAATCTCG-3' (SEQ ID NO:157) for murine TLR9 according to the manufacturer's protocol.

For the stimulation of mTLR9 variant, mTLR9-PHmut, 293 cells were transiently transfected with mTLR9 or mTLR9-PHmut and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 22**). 48 hours after stimulation 15 supernatant was harvested and IL-8 production was measured by ELISA. For the stimulation of hTLR9 variant, hTLR9-PHmut, 293 cells were transiently transfected with hTLR9 or hTLR9-PHmut and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 23**). 48 hours after stimulation supernatant was harvested 20 and IL-8 production was measured by ELISA. These data demonstrate that TLR9 activity can be destroyed by the Pro to His mutation. This mutation has the potential to be used as a dominant negative to block TLR9 activity thus a genetic variant could compete for ligand or signaling partners and disrupt signaling.

**Example 13. Exchange of the TIR-domain between murine and human TLR9 (hTLR9-TIRm, mTLR9-TIRh)**

Toll-like receptors have a cytoplasmic Toll/IL-1 receptor (TIR) homology domain that initiates signaling after binding of the adapter molecule MyD88. Medzhitov R et al., *Mol Cell* 2:253-8 (1998); Kopp EB et al., *Curr Opin Immunol* 11:15-8 (1999). This is also true for TLR9. To generate molecules consisting of human extracellular TLR9 and murine TIR 30 domain (hTLR9-TIRm) or murine extracellular TLR9 and human TIR domain (mTLR9-TIRh), the following approach was chosen. Through site-specific mutagenesis a ClaI

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restriction site was introduced in human and murine TLR9. For human TLR9 the DNA sequence 5'-GGCCTCAGCATCTT-3' (3026-3040, SEQ ID NO:158) was mutated to 5'-GGCCTATCGATTTT-3' (SEQ ID NO:159), introducing a Clal site (underlined in the sequence) but leaving the amino acid sequence (GLSIF, aa 798-802) unchanged. For murine  
5 TLR9 the DNA sequence 5'-GCCGTAGCATCTTC-3' (2434-2447, SEQ ID NO:160) was mutated to 5'-GGCCTATCGATTTT-3' (SEQ ID NO:161), introducing a Clal site and creating the amino acid sequence (GLSIF, aa 799-803) which differs in one position (aa 800) from the wildtype murine TLR9 sequence (GRSIF, aa 799-803) but is identical to the human sequence.

10 **hTLR9-TIRm.** The primers used for human TLR9 were 5'-CAGCTCCAGGCCTATCGATTGCACAGGACC-3' (SEQ ID NO:162) and 5'-GGTCCTGTGCAAAATCGATAAGGCCCTGGAGCTG-3' (SEQ ID NO:163). For creating an expression vector containing the extracellular portion of human TLR9 connected to the murine TIR domain, the human expression vector was cut with Clal and limiting amounts of  
15 EcoRI and the fragment coding for the murine TIR domain generated by a Clal and EcoRI digestion of murine TLR9 expression vector was ligated in the vector fragment containing the extracellular portion of hTLR9. Transfection into *E.coli* yielded the expression vector hTLR9-TIRm (human extracellular TLR9-murine TIR-domain).

20 **mTLR9-TIRh.** The primers used for murine TLR9 were 5'-CAGCTGCAGGCCTATCGATTTCGCACAGGACC-3' (SEQ ID NO:164) and 5'-GGTCCTGTGCGAAAATCGATAAGGCCCTGCAGCTG-3' (SEQ ID NO:165). For creating an expression vector containing the extracellular portion of murine TLR9 connected to the human TIR domain, the murine expression vector was cut with Clal and limiting amounts of EcoRI and the fragment coding for the human TIR domain generated by a Clal and EcoRI  
25 digestion of human TLR9 expression vector was ligated in the vector fragment containing the extracellular portion of mTLR9. Transfection into *E.coli* yielded the expression vector mTLR9-TIRh (murine extracellular TLR9-human TIR-domain).

For the stimulation of the mTLR9 variant, mTLR9-TIRh, 293 cells were transiently transfected with mTLR9 or mTLR9-TIRh and stimulated after 16 hours with ODN 2006 and  
30 ODN 1668 at concentrations indicated (**Figure 24**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. For the stimulation of the

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hTLR9 variant, hTLR9-TIRm, 293 cells were transiently transfected with hTLR9 or hTLR9-TIRm and stimulated after 16 hours with ODN 2006 and ODN 1668 at concentrations indicated (**Figure 25**). 48 hours after stimulation supernatant was harvested and IL-8 production was measured by ELISA. Replacement of the murine TLR9-TIR domain with 5 human does not significantly affect mTLR9 activity. Replacement of the human TLR9-TIR with murine however appears to have a negative effect on hTLR9. These data demonstrate that manipulations could be made to influence TLR9 activities.

**Example 14. TLR9-fusion protein with green-fluorescent-protein (hTLR9-GFP, 10 mTLR9-GFP)**

Human and murine TLR9 were individually cloned into the vector pEGFP-N1 (Clontech, Palo Alto, CA, USA) to create expression vectors encoding human and murine fusion proteins consisting of an N-terminal TLR9 protein fused to C-terminal green-fluorescent protein (GFP). These constructs can be used to trace TLR9 localization and 15 expression. Such detections can be used for staining in FACS analysis, confocal microscopy and Western blot, or for purification of polypeptides and subsequent antibody production.

**Example 15. TLR9-fusion protein with FLAG-peptide (hTLR9-FLAG, mTLR9-FLAG)**

Human and murine TLR9 were individually cloned into the vector pFLAG-CMV-1 20 (Sigma, St. Louis, MO, USA) to create expression vectors encoding human and murine fusion proteins consisting of an N-terminal leader peptide (protrypsin, which is cleaved intracellularly during processing of the protein), FLAG-peptide (DYKDDDDK) and TLR9 protein which does not contain its own signal peptide. These constructs can be used to trace 25 TLR9 localization and expression, e.g., using anti-FLAG antibodies. Such detections can be used for staining in FACS analysis, confocal microscopy and Western blot, or for purification of polypeptides and subsequent antibody production.

**Example 16. Method of cloning human TLR7**

Two accession numbers in the GenBank database, AF245702 and AF240467, describe 30 the DNA sequence for human TLR7. To create an expression vector for human TLR7, human TLR7 cDNA was amplified from a cDNA made from human peripheral mononuclear

blood cells (PBMC) using the primers 5'-CACCTCTCATGCTCTGCTCTTC-3' (SEQ ID NO:166) and 5'-GCTAGACCGTTCCCTGAACACCTG-3' (SEQ ID NO:167). The fragment was cloned into pGEM-T Easy vector (Promega), cut with the restriction enzyme NotI and ligated into a NotI-digested pCDNA3.1 expression vector (Invitrogen). The insert 5 was fully sequenced and translated into protein. The cDNA sequence for hTLR7 is SEQ ID NO:168, is presented in Table 6. The open reading frame starts at base 124, ends at base 3273, and codes for a protein of 1049 amino acids. SEQ ID NO:169 (Table 7), corresponding to bases 124-3273 of SEQ ID NO:168 (Table 6), is the coding region for the polypeptide of SEQ ID NO:170 (Table 8).

10 The protein sequence of the cloned hTLR7 cDNA matches the sequence described under the GenBank accession number AF240467. The sequence deposited under GenBank accession number AF245702 contains two amino acid changes at position 725 (L to H) and 738 (L to P).

15 **Table 6. cDNA Sequence for Human TLR7 (5' to 3'; SEQ ID NO:168)**

	agctggctag cgtttaaacg ggccctctag actcgagcgg ccgcgaattc actagtgtatt	60
	cacccatgttcat gctctgctct cttcaaccagg acctctacat tccatgggg aagaagacta	120
	aaaatggtgt ttccaatgtg gacactgaag agacaaattc ttatcctttt taacataatc	180
	ctaatttcca aactcccttg ggcttagatgg tttcctaaaa ctctgccctg tgatgtcact	240
20	ctggatgttc caaagaacca tgtgtatgtg gactgcacag acaagcattt gacagaaatt	300
	cctggaggta ttcccacgaa caccacgaac ctcaccctca ccattaacca cataccagac	360
	atctccccag cgtcctttca cagactggac catctggtag agatcgattt cagatgcaac	420
	tgtgtaccta ttccactggg gtcaaaaaac aacatgtgca tcaagaggct gcagattaaa	480
	cccagaagct ttagtggact cacttattta aaatccctt acctggatgg aaaccagcta	540
25	ctagagatac cgcagggcct cccgccttagc ttacagcttc tcagccttga ggccaacaac	600
	atcttttcca tcagaaaaga gaatctaaca gaactggcca acatagaaat actctacact	660
	ggccaaaaact gttattatcg aaatccttgt tatgtttcat attcaataga gaaagatgcc	720
	ttcctaaact tgacaaaagtt aaaagtgctc tccctgaaag ataacaatgt cacagccgtc	780
	cctactgttt tgccatctac tttaacagaa ctatatctt acaacaacat gattgcaaaa	840
30	atccaagaag atgattttaa taacctcaac caattacaaa ttcttgacct aagtggaaat	900
	tgccctcggtt gttataatgc cccatttcct tggcgccgt gtaaaaataa ttctccctta	960
	cagatccctg taaatgcttt tgatgcgtg acagaattaa aagtttacg tctacacagt	1020
	aactctcttcc agcatgtgcc cccaaagatgg tttaagaaca tcaacaaact ccaggaactg	1080
	gatctgtccc aaaacttctt ggccaaagaa attggggatg ctaaatttctt gcattttctc	1140
35	cccagcctca tccaatttggaa tctgtctttc aattttgaac ttcaggtctt tcgtgcattt	1200
	atgaatcttat cacaaggcatt ttcttcactg aaaagcctga aaattctgcg gatcagagga	1260
	tatgtcttta aagagttgaa aagcttaac ctctcgccat tacataatct tcaaaatctt	1320

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	gaagttcttg atcttggcac taactttata aaaattgcta acctcagcat gtttaaacaa	1380
	tttaaaagac tgaaaagtcat agatcttca gtgaataaaa tatcacccttc aggagattca	1440
	agtgaagttg gcttctgctc aaatgccaga acttctgttag aaagttatga accccaggtc	1500
	ctggAACAAAT tacattattt cagatatgtat aagtatgcaa ggagttgcag attcaaaaac	1560
5	aaagaggctt ct当地atgtc tgtaatgaa agctgctaca agtatggca gaccttggat	1620
	ctaaatgtaaaa atagtatatt ttttgtcaag tcctctgatt tt当地agcatct tt当地tttctc	1680
	aaatgcctga atctgtcagg aaatctcatt agccaaactc ttaatggcag tgaattccaa	1740
	ccttagcag agctgagata tttggacttc tccaacaacc ggcttgattt actccattca	1800
	acagcattt aagagcttca caaactggaa gttctggata taagcagtaa tagccattat	1860
10	tttcaatcag aaggaattac tc当地atgcta aactttacca agaacctaaa ggttctgcag	1920
	aaactgatga tgaacgacaa tgacatctct tc当地ccacca gcaggaccat ggagagtgag	1980
	tctcttagaa ct当地tggaaat cagagggaaat cacttagatg tttatggag agaagggtat	2040
	aacagatact tacaattatt caagaatctg ct当地aaattag aggaatttaga catctctaaa	2100
	aattccctaa gtttcttgc当地 ttctggagtt tttgatgta tgc当地ccaaa tctaaagaat	2160
15	ctctcttgg ccaaaaatgg gctcaaatact ttc当地gttggaa agaaaactcca gt当地ctaaag	2220
	aaactggaaa ct当地ggaccc tggccacaac caactgacca ct当地ccctga gagattatcc	2280
	aactgttcca gaagcctcaa gaatctgatt ct当地agaata atcaaatacg gagtctgc当地	2340
	aagtatttcc tacaagatgc ct当地caggat cgatatctgg atctcagctc aaataaaaatc	2400
	cagatgatcc aaaagaccag ct当地ccagaa aatgtcctca acaatctgaa gatgttgc当地	2460
20	ttgc当地atcata atcggtttct gt当地ccctgt gatgctgtgt ggttgtctg gt当地ggtaac	2520
	catacggagg tgactattcc tt当地ctggcc acagatgtga ct当地gttggg gccaggagca	2580
	cacaaggggcc aaagtgtgat ct当地ctggat ct当地tacacct gt当地gatgtaac	2640
	ctgattctgt tctc当地tccata catatctgta tctcttccat tcatggat gatgacagca	2700
	agtcacctct atttctggat tgc当地gttggat atttaccatt tctgtaaggc caagataaaag	2760
25	gggtatcagc gt当地taatatc accagactgt tgctatgatg ct当地tattgt gt当地gacact	2820
	aaagaccagg ct当地tgaccga gt当地ggtttg gctgagctgg tggccaaact ggaagacccc	2880
	agagagaaaac attttaattt atgtctcgag gaaagggact ggttaccagg gc当地ccagtt	2940
	ctggAAAACCCTCAGAG catacagctt agcaaaaaga cagtgatgt gatgacagac	3000
	aagtatgcaa agactgaaaaa tt当地taagata gcattttact tgc当地ccatca gaggctcatg	3060
30	gatgaaaaag tt当地atgtgat tatcttgc当地 tt当地cttgc当地 agc当地tttca gaagtc当地aaag	3120
	ttc当地ctccagc tccggAAAAG gctctgtggg agttctgtcc tt当地gttggcc aacaaaccgg	3180
	caagctcacc catacttctg gcagtgatctaa aagaacgccc tggccacaga caatcatgtg	3240
	gcctatagtc aggtgttcaa ggaaacggc tagaatcgaa tt当地ccgc当地 cc当地actgtg	3300
	ctggatatact gc当地gaattcc accacactgg acttagtggat cc当地gctcg当地 taccaaagctt	3360
35	aagtttaaac cgc	3373

**Table 7. Coding Region for Human TLR7 (5' to 3'; SEQ ID NO:169)**

```

atggtgtttc caatgtggac actgaagaga caaattctta tccttttaa cataatccta 60
atttccaaac tccttgggc tagatggttt cctaaaactc tgccctgtga tgtcaactcg 120
40 gatgttccaa agaaccatgt gatcggtggac tgcacagaca agcatttgac agaaattcct 180
qqaggtattc ccacqaacac cacqaacctc accctcacca ttaaccacat accaqacatc 240

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	tccccagcgt	cctttcacag	actggaccat	ctggtagaga	tcgatttcag	atgcaactgt	300
	gtaccttattc	caactggggtc	aaaaaaacaac	atgtgcata	agaggctgca	gattaaaccc	360
	agaagcttta	gtggactcac	ttatttaaaa	tcccttacc	tggatggaaa	ccagctacta	420
	gagataccgc	agggcctccc	gcctagctta	cagcttctca	gccttgaggc	caacaacatc	480
5	ttttccatca	gaaaagagaa	tctaacagaa	ctggccaaca	tagaaatact	ctacctggc	540
	caaaaactgtt	attatcgaaa	tccttggtat	gtttcatatt	caatagagaa	agatgcctc	600
	ctaaacttga	caaagtaaa	agtgcctcc	ctgaaagata	acaatgtcac	agccgtccct	660
	actgtttgc	catctacttt	aacagaacta	tatctctaca	acaacatgtat	tgcaaaaatc	720
	caagaagatg	attttaataa	cctcaaccaa	ttacaaattc	ttgacctaag	tggaaattgc	780
10	cctcgttgtt	ataatgcccc	atttccttgt	gcgccgtgta	aaaataattc	tcccctacag	840
	atccctgtaa	atgcctttga	tgcgctgaca	gaattaaaaag	ttttacgtct	acacagtaac	900
	tctcttcagc	atgtgcffff	aagatggttt	aagaacatca	acaaaactcca	ggaactggat	960
	ctgtcccaaa	acttcttggc	caaagaaaatt	ggggatgcta	aatttctgca	ttttctcccc	1020
	agcctcatcc	aatttggatct	gtcttcaat	tttgaacttc	aggctatcg	tgcatctatg	1080
15	aatctatcac	aagcattttc	ttcactgaaa	agcctgaaaa	ttctgcggat	cagaggat	1140
	gtctttaaag	agttgaaaag	ctttaacctc	tcgccattac	ataatcttca	aaatctgaa	1200
	gttcttgatc	ttggcactaa	ctttataaaa	attgctaacc	tcagcatgtt	taaacaattt	1260
	aaaagactga	aagtcataga	tcttcagtg	aataaaaat	cacccctcagg	agattcaagt	1320
	gaagttggct	tctgctcaaa	tgccagaact	tctgtagaaa	gttatgaacc	ccaggtcctg	1380
20	gaacaattac	attatttcag	atatgataag	tatgcaagga	gttgcagatt	caaaaacaaa	1440
	gaggcttctt	tcatgtctgt	taatgaaagc	tgctacaagt	atgggcagac	cttggatcta	1500
	agtaaaaaata	gtatattttt	tgtcaagtcc	tctgattttc	agcatcttc	tttcctcaaa	1560
	tgcctgaatc	tgtcaggaaa	tctcattagc	caaactctta	atggcagtga	attccaacct	1620
	ttagcagagc	ttagatattt	ggacttctcc	aacaaccggc	ttgatttact	ccattcaaca	1680
25	gcatttgaag	agcttcacaa	actggaagtt	ctggatataa	gcagtaatag	ccattatttt	1720
	caatcagaag	gaattactca	tatgctaaac	tttaccaaga	acctaaaggt	tctgcagaaa	1800
	ctgatgatga	acgacaatga	catctttcc	tccaccagca	ggaccatgga	gagtgagtct	1860
	cttagaactc	tgaaatttcag	aggaaatcac	ttagatgttt	tatggagaga	aggtgataac	1920
	agataactac	aattattcaa	gaatctgcta	aaattagagg	aattagacat	ctctaaaaat	1980
30	tccctaagtt	tcttgccttc	tggagttttt	gatggatgc	ctccaaatct	aaagaatctc	2040
	tctttggcca	aaaatgggct	caaatcttc	agttgaaaga	aactccagt	tctaaagaac	2100
	ctggaaactt	tggacctcag	ccacaaccaa	ctgaccactg	tccctgagag	attatccaac	2160
	tgttccagaa	gcctcaagaa	tctgattctt	aagaataatc	aaatcaggag	tctgacgaag	2220
	tattttctac	aagatgcctt	ccagttgcga	tatctggatc	tcagctcaaa	taaaatccag	2280
35	atgatccaaa	agaccagctt	cccagaaaaat	gtcctcaaca	atctgaagat	gttgctttg	2340
	catcataatc	ggtttctgtg	cacctgtgat	gttgtgtggt	ttgtctgggt	ggttaaccat	2400
	acggagggtga	ctattcctta	cctggccaca	gatgtgactt	gtgtggggcc	aggagcacac	2460
	aaggggccaaa	gtgtgatctc	cctggatctg	tacaccctgtg	agtttagatct	gactaacctg	2520
	attctgttct	cactttccat	atctgtatct	ctctttctca	tggtgatgat	gacagcaagt	2580
40	cacctcttatt	tctggatgt	gtggatattt	taccatttct	gtaaggccaa	gataaagggg	2640
	tatcagcgtc	taatatcacc	agactgttgc	tatgatgctt	ttattgtgt	tgacactaaa	2700
	gaccctcg	tgaccgagtg	ggttttggct	gagctggtgg	ccaaactgg	agacccaaga	2760

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5	gagaaacatt ttaatttatg tctcgaggaa agggacttgt taccaggca gccagttctg gaaaaacctt cccagagcat acagcttagc aaaaagacag tgtttgtat gacagacaag tatgcaaaga ctgaaaattt taagatagca ttttacttgtt cccatcagag gtcatggat gaaaaaagtgt atgtgattat cttgatattt cttgagaagc cttttcagaa gtccaagttc ctccagctcc ggaaaaaggct ctgtggaggt tctgtccttg agtggccaac aaacccgcaa gctcacccat acttctggca gtgtctaaag aacgccttgg ccacagacaa tcatgtggcc tataqtcaagg tqttcaagqa aacqqtc	2820 2880 2940 3000 3060 3120 3147
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**Table 8. Amino Acid Sequence of Human TLR7**

10		MVFPWMWTLKRQILILFNIILISKLLGARWFPKTLPCDVTLDVPKNHVIVDCTDKHLTEIP	60
	hTLR7.pep	MVFPWMWTLKRQILILFNIILISKLLGARWFPKTLPCDVTLDVPKNHVIVDCTDKHLTEIP	60
	AF245702.pep	MVFPWMWTLKRQILILFNIILISKLLGARWFPKTLPCDVTLDVPKNHVIVDCTDKHLTEIP	60
15			
	AF240467.pep	GGIPTNTTNLTLTINHI PDISPASFHRLDHLVEIDFRCNCVPIPLGSKNNMCIKRLQIKP	120
	hTLR7.pep	GGIPTNTTNLTLTINHI PDISPASFHRLDHLVEIDFRCNCVPIPLGSKNNMCIKRLQIKP	120
	AF245702.pep	GGIPTNTTNLTLTINHI PDISPASFHRLDHLVEIDFRCNCVPIPLGSKNNMCIKRLQIKP	120
20			
	AF240467.pep	RSFSGLTYLKSLYLDGNQILLEIPQGLPPSLQQLSLEANNIFSIRKENLTELANIEILYLG	180
	hTLR7.pep	RSFSGLTYLKSLYLDGNQILLEIPQGLPPSLQQLSLEANNIFSIRKENLTELANIEILYLG	180
	AF245702.pep	RSFSGLTYLKSLYLDGNQILLEIPQGLPPSLQQLSLEANNIFSIRKENLTELANIEILYLG	180
25			
	AF240467.pep	QNCYYRNPCYVSYISIEKDAFLNLTKLKVLSLKDNNTAVPTVLPSTLTLYLYNNMIAKI	240
	hTLR7.pep	QNCYYRNPCYVSYISIEKDAFLNLTKLKVLSLKDNNTAVPTVLPSTLTLYLYNNMIAKI	240
	AF245702.pep	QNCYYRNPCYVSYISIEKDAFLNLTKLKVLSLKDNNTAVPTVLPSTLTLYLYNNMIAKI	240
30			
	AF240467.pep	QEDDFNNLNQLQILDLSGNCPRCYNAPFPCAPCKNNSPLOQIPVNNAFDALTELKVRLHSN	300
	hTLR7.pep	QEDDFNNLNQLQILDLSGNCPRCYNAPFPCAPCKNNSPLOQIPVNNAFDALTELKVRLHSN	300
	AF245702.pep	QEDDFNNLNQLQILDLSGNCPRCYNAPFPCAPCKNNSPLOQIPVNNAFDALTELKVRLHSN	300
35			
	AF240467.pep	SLQHVPWRWFKNINKLQELDLSQNFLAKEIGDAKFLHFLPSLIQLDLSFNFELQVYRASM	360
	hTLR7.pep	SLQHVPWRWFKNINKLQELDLSQNFLAKEIGDAKFLHFLPSLIQLDLSFNFELQVYRASM	360
	AF245702.pep	SLQHVPWRWFKNINKLQELDLSQNFLAKEIGDAKFLHFLPSLIQLDLSFNFELQVYRASM	360
40			
	AF240467.pep	NLSQAFSSLKSLKILRIRGYVFKEFKSFNLSPLNHNLQNLEVLDLGTNFIFKIANLSMFQF	420
	hTLR7.pep	NLSQAFSSLKSLKILRIRGYVFKEFKSFNLSPLNHNLQNLEVLDLGTNFIFKIANLSMFQF	420
	AF245702.pep	NLSQAFSSLKSLKILRIRGYVFKEFKSFNLSPLNHNLQNLEVLDLGTNFIFKIANLSMFQF	420
45			

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	AF240467.pep	KRLKVIDLSVNKISPSGDSSEVGFCNSARTSVESYEPQVLEQLHYFRYDKYARSCRFKNK	480
	hTLR7.pep	KRLKVIDLSVNKISPSGDSSEVGFCNSARTSVESYEPQVLEQLHYFRYDKYARSCRFKNK	480
	AF245702.pep	KRLKVIDLSVNKISPSGDSSEVGFCNSARTSVESYEPQVLEQLHYFRYDKYARSCRFKNK	480
5	.	.	540
	AF240467.pep	EASFMSVNESCYKYGQTLDLSKNSIFFVKSSDFQHLSFLKCLNLSGNLISQTLNGSEFQP	540
	hTLR7.pep	EASFMSVNESCYKYGQTLDLSKNSIFFVKSSDFQHLSFLKCLNLSGNLISQTLNGSEFQP	540
	AF245702.pep	EASFMSVNESCYKYGQTLDLSKNSIFFVKSSDFQHLSFLKCLNLSGNLISQTLNGSEFQP	540
10	.	.	600
	AF240467.pep	LAEILYLDLFSNNRLDLLHSTAFEELHKLEVLDISSLNSHYFQSEGITHMLNFTKNLKVLOK	600
	hTLR7.pep	LAEILYLDLFSNNRLDLLHSTAFEELHKLEVLDISSLNSHYFQSEGITHMLNFTKNLKVLOK	600
	AF245702.pep	LAEILYLDLFSNNRLDLLHSTAFEELHKLEVLDISSLNSHYFQSEGITHMLNFTKNLKVLOK	600
15	.	.	660
	AF240467.pep	LMMNDNDISSLRTMEESLRTLEFRGNHLDVLWREGDNRYLQLFKNLLKLEELDISKN	660
	hTLR7.pep	LMMNDNDISSLRTMEESLRTLEFRGNHLDVLWREGDNRYLQLFKNLLKLEELDISKN	660
	AF245702.pep	LMMNDNDISSLRTMEESLRTLEFRGNHLDVLWREGDNRYLQLFKNLLKLEELDISKN	660
20	.	.	720
	AF240467.pep	SLSFLPSGVFDGMPPNLKNLSLAKNGLKSFSWKKLQCLKNLETLDLSHNQLTTVPERLSN	720
	hTLR7.pep	SLSFLPSGVFDGMPPNLKNLSLAKNGLKSFSWKKLQCLKNLETLDLSHNQLTTVPERLSN	720
	AF245702.pep	SLSFLPSGVFDGMPPNLKNLSLAKNGLKSFSWKKLQCLKNLETLDLSHNQLTTVPERLSN	720
25	.	.	780
	AF240467.pep	CSRS <span style="text-decoration: underline;">L</span> KNL <span style="text-decoration: underline;">L</span> IKNNQIRSLTKYFLQDAFQLRYLDLSSNKIQMIQKTSFPENVLNNLKMLLL	780
	hTLR7.pep	CSRS <span style="text-decoration: underline;">L</span> KNL <span style="text-decoration: underline;">L</span> IKNNQIRSLTKYFLQDAFQLRYLDLSSNKIQMIQKTSFPENVLNNLKMLLL	780
	AF245702.pep	CSRS <span style="text-decoration: underline;">H</span> KNL <span style="text-decoration: underline;">L</span> IKNNQIRSP <span style="text-decoration: underline;">T</span> KYFLQDAFQLRYLDLSSNKIQMIQKTSFPENVLNNLKMLLL	780
30	.	.	840
	AF240467.pep	HHNRFLCTCDAVWFVWWVNHTEVТИPYLATDVTCPGGAHKGQSVISLDLYTCELDLTNL	840
	hTLR7.pep	HHNRFLCTCDAVWFVWWVNHTEVТИPYLATDVTCPGGAHKGQSVISLDLYTCELDLTNL	840
	AF245702.pep	HHNRFLCTCDAVWFVWWVNHTEVТИPYLATDVTCPGGAHKGQSVISLDLYTCELDLTNL	840
35	.	.	900
	AF240467.pep	ILF <span style="text-decoration: underline;">S</span> LSISVSL <span style="text-decoration: underline;">L</span> FMVMMTASHLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTK	900
	hTLR7.pep	ILF <span style="text-decoration: underline;">S</span> LSISVSL <span style="text-decoration: underline;">L</span> FMVMMTASHLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTK	900
	AF245702.pep	ILF <span style="text-decoration: underline;">S</span> LSISVSL <span style="text-decoration: underline;">L</span> FMVMMTASHLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFIVYDTK	900
40	.	.	960
	AF240467.pep	DPAVTEWVL <span style="text-decoration: underline;">A</span> ELVAKLEDPREKH <span style="text-decoration: underline;">F</span> NLC <span style="text-decoration: underline;">E</span> ERDWLP <span style="text-decoration: underline;">G</span> QP <span style="text-decoration: underline;">V</span> LEN <span style="text-decoration: underline;">L</span> SQSIQLSKKT <span style="text-decoration: underline;">V</span> FVMTDK	960
	hTLR7.pep	DPAVTEWVL <span style="text-decoration: underline;">A</span> ELVAKLEDPREKH <span style="text-decoration: underline;">F</span> NLC <span style="text-decoration: underline;">E</span> ERDWLP <span style="text-decoration: underline;">G</span> QP <span style="text-decoration: underline;">V</span> LEN <span style="text-decoration: underline;">L</span> SQSIQLSKKT <span style="text-decoration: underline;">V</span> FVMTDK	960
	AF245702.pep	DPAVTEWVL <span style="text-decoration: underline;">A</span> ELVAKLEDPREKH <span style="text-decoration: underline;">F</span> NLC <span style="text-decoration: underline;">E</span> ERDWLP <span style="text-decoration: underline;">G</span> QP <span style="text-decoration: underline;">V</span> LEN <span style="text-decoration: underline;">L</span> SQSIQLSKKT <span style="text-decoration: underline;">V</span> FVMTDK	960
45	.	.	1020
	AF240467.pep	YAKTENFKIAFYL <span style="text-decoration: underline;">S</span> HQR <span style="text-decoration: underline;">L</span> MDEKVDVII <span style="text-decoration: underline;">I</span> LF <span style="text-decoration: underline;">E</span> KPFQ <span style="text-decoration: underline;">K</span> SKFLQ <span style="text-decoration: underline;">L</span> R <span style="text-decoration: underline;">K</span> LC <span style="text-decoration: underline;">G</span> SSV <span style="text-decoration: underline;">L</span> EWP <span style="text-decoration: underline;">T</span> N <span style="text-decoration: underline;">P</span> Q	1020

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hTLR7.pep	YAKTENFKIAFYLSHQRLMDEKVDVIIILIFLEKPFQKSFLQLRKRLCGSSVLEWPTNPQ	1020
AF245702.pep	YAKTENFKIAFYLSHQRLMDEKVDVIIILIFLEKPFQKSFLQLRKRLCGSSVLEWPTNPQ	1020
5	AF240467.pep AHPYFWQCLKNALATDNHVAYSQVFKEIV	1080
	hTLR7.pep AHPYFWQCLKNALATDNHVAYSQVFKEIV	1049
	AF245702.pep AHPYFWQCLKNALATDNHVAYSQVFKEIV	1049

In Table 8 the sequences are assigned as follows: hTLR7.pep, SEQ ID NO:170;  
 10 AF240467.pep, SEQ ID NO:171; AF245702.pep, SEQ ID NO:172.

#### **Example 17. Method of cloning the murine TLR7**

Alignment of human TLR7 protein sequence with mouse EST database using tfasta yielded 4 hits with mouse EST sequences bb116163, aa266744, bb210780 and aa276879.

15 Two primers were designed that bind to aa266744 sequence for use in a RACE-PCR to amplify 5' and 3' ends of the murine TLR7 cDNA. The library used for the RACE PCR was a mouse spleen marathon-ready cDNA commercially available from Clontech. A 5' fragment with a length of 3000 bp obtained by this method was cloned into Promega pGEM-T Easy vector. After sequencing of the 5' end, additional primers were designed for amplification of  
 20 the complete murine TLR7 cDNA. The primer for the 5' end was obtained from the sequence of the 5' RACE product whereas the primer for the 3' end was selected from the mouse EST sequence aa266744.

Three independent PCR reactions were set up using a murine macrophage RAW264.7 (ATCC TIB-71) cDNA as a template with the primers 5'-  
 25 CTCCTCCACCAAGACCTCTTGATTCC-3' (SEQ ID NO:208) and 5'- CAAGGCATGTCCTAGGTGGTGACATTC-3' (SEQ ID NO:209). The resulting amplification products were cloned into pGEM-T Easy vector and fully sequenced (SEQ ID NO:173; Table 9). The open reading frame of mTLR7 (SEQ ID NO:174; Table 10) starts at base 49, ends at base 3201 and codes for a protein of 1050 amino acids (SEQ ID NO:175; Table 11). To create an expression vector for murine TLR7 cDNA, pGEM-T Easy vector plus mTLR7 insert was cut with NotI, the fragment isolated and ligated into a NotI digested pCDNA3.1 expression vector (Invitrogen).

**Table 9. cDNA Sequence for Murine TLR7 (5' to 3'; SEQ ID NO:173)**

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	ATTCTCCTCC ACCAGACCTC TTGATTCCAT TTTGAAAGAA AACTGAAAAT GGTGTTTCG	60
	ATGTGGACAC GGAAGAGACA AATTTGATC TTTTAAATA TGCTCTTAGT TTCTAGAGTC	120
	TTGGGTTTC GATGGTTTC TAAAACCTCA CCTTGTAAG TTAAAGTAAA TATCCCAGAG	180
	GCCCATGTGA TCGTGGACTG CACAGACAAG CATTGACAG AAATCCCTGA GGGCATTCCC	240
5	ACTAACACCA CCAATCTTAC CCTTACCATC AACCACATAC CAAGCATCTC TCCAGATTCC	300
	TTCCGTAGGC TGAACCATCT GGAAGAAATC GATTTAAGAT GCAATTGTGT ACCTGTTCTA	360
	CTGGGGTCCA AAGCCAATGT GTGTACCAAG AGGCTGCAGA TTAGACCTGG AAGCTTAGT	420
	GGACTCTCTG ACTTAAAAGC CCTTACCTG GATGGAAACC AAATTCTGGA GATACCACAG	480
	GATCTGCCAT CCAGCTTACA TCTTCTGAGC CTTGAGGCTA ACAACATCTT CTCCATCACG	540
10	AAGGAGAATC TAACAGAACT GGTCAACATT GAAACACTCT ACCTGGGTCA AAACTGTTAT	600
	TATCGAAATC CTTGCAATGT TTCTTATTCT ATTGAAAAAG ATGTTTCCT AGTTATGAGA	660
	AATTGAGG TTCTCTCACT AAAAGATAAC AATGTCACAG CTGCCCCAC CACTTGCCA	720
	CCTAATTAC TAGAGCTCTA TCTTATAAC AATATCATTAA AGAAAATCCA AGAAAATGAT	780
	TTTAATAAACC TCAATGAGTT GCAAGTTCTT GACCTAAGTG GAAATTGCC TCGATGTTAT	840
15	AATGTCCCCT ATCCGTGTAC ACCGTGTGAA AATAATTCCC CCTTACAGAT CCATGACAAT	900
	GCTTTCAATT CATTGACAGA ATTAAAAGTT TTACGTTTAC ACAGTAATT TCCTTCAGCAT	960
	GTGCCCCCAA CATGGTTAA AACATGAGA AACCTCCAGG AACTAGACCT CTCCCAAAAC	1020
	TACTTGGCCA GAGAAATTGA GGAGGCCAAA TTTTGCATT TTCTTCCCAA CCTTGTGAG	1080
	TTGGATTTT CTTCAATTAA TGAGCTGCAG GTCTACCATG CATCTATAAC TTTACCACAT	1140
20	TCACTCTCTT CATTGGAAAAA CTTGAAAATT CTGCGTGTCA AGGGGTATGT CTTTAAAGAG	1200
	CTGAAAAACT CCAGTCTTTC TGTATTGCAC AAGCTTCCA GGCTGGAAGT TCTTGACCTT	1260
	GGCACTAACT TCATAAAAAT TGCTGACCTC AACATATTCA AACATTTCGA AAACCTCAAA	1320
	CTCATAGACC TTTCAGTGA TAAGATATCT CCTTCAGAAG AGTCAAGAGA AGTTGGCTTT	1380
	TGTCCTAATG CTCAAACTTC TGTAGACCGT CATGGGCCAGG AGGTCTTGA GGCCTTACAC	1440
25	TATTTCCGAT ACGATGAATA TGCACGGAGC TGCAGGTTCA AAAACAAAGA GCCACCTTCT	1500
	TTCTTGCCCTT TGAATGCAGA CTGCCACATA TATGGCAGA CCTTAGACTT AAGTAGAAAT	1560
	AACATATTAA TTATTAAACC TTCTGATTTT CAGCATCTT CATTCTCAA ATGCCTCAAC	1620
	TTATCAGGAA ACACCATTGG CCAAACCTTT AATGGCAGTG AACCTCTGGCC GTTGAGAGAG	1680
	TTGCGGTACT TAGACTTCTC CAACAACCGG CTTGATTAC TCTACTCAAC AGCCTTGAA	1740
30	GAGCTCCAGA GTCTGAAAGT TCTGGATCTA AGTAGTAACA GCCACTATT TCAAGCAGAA	1800
	GGAATTACTC ACATGCTAAA CTTTACCAAG AAATTACGGC TTCTGGACAA ACTCATGATG	1860
	AATGATAATG ACATCTCTAC TTCGGCCAGC AGGACCATGG AAAGTGACTC TCTTCGAATT	1920
	CTGGAGTTCA GAGGCAACCA TTTAGATGTT CTATGGAGAG CGGGTGATAA CAGATACTG	1980
	GACTTCTTCA AGAATTGTT CAATTAGAG GTATTAGATA TCTCCAGAAA TTCCCTGAAT	2040
35	TCCTTGCCCTC CTGAGGTTTT TGAGGGTATG CCGCCAAATC TAAAGAATCT CTCCCTGGCC	2100
	AAAAATGGGC TCAAATCTTT CTTTGGGAC AGACTCCAGT TACTGAAGCA TTTGGAAATT	2160
	TTGGACCTCA GCCATAACCA GCTGACAAAA GTACCTGAGA GATTGGCCAA CTGTTCCAAA	2220
	AGTCTCACAA CACTGATTCT TAAGCATAAT CAAATCAGGC AATTGACAAA ATATTTCTA	2280
	GAAGATGCTT TGCAATTGCG CTATCTAGAC ATCAGTTCAA ATAAAATCCA GGTCAATTCA	2340
40	AAGACTAGCT TCCCAGAAAAA TGTCTCAAC AATCTGGAGA TGTGGTTTT ACATCACAAT	2400
	CGCTTCTTT GCAACTGTGA TGCTGTGTGG TTTGTCTGGT GGGTTAACCA TACAGATGTT	2460
	ACTATTCCAT ACCTGGCCAC TGATGTGACT TGTGTAGGTC CAGGAGCACA CAAAGGTCAA	2520

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	AGTGTCAAT AT CCCTTGATCT GTATACGTGT GAGTTAGATC TCACAAACCT GATTCTGTTC	2580
	TCAGTTCCA TATCATCAGT CCTCTTCTT ATGGTAGTTA TGACAACAAG TCACCTCTT	2640
	TTCTGGGATA TGTGGTACAT TTATTATTT TGAAAGCAA AGATAAAGGG GTATCAGCAT	2700
	CTGCAATCCA TGGAGTCTTG TTATGATGCT TTTATTGTGT ATGACACTAA AACTCAGCT	2760
5	GTGACAGAAT GGTTTTGCA GGAGCTGGTG GCAGAAATTGG AAGATCCAAG AGAAAAACAC	2820
	TTCAATTGTG TCTAGAAGA AAGAGACTGG CTACCAGGAC AGCCAGTTCT AGAAAACCTT	2880
	TCCCAGAGCA TACAGCTCAG CAAAAAGACA GTGTTGTGA TGACACAGAA ATATGCTAAG	2940
	ACTGAGAGTT TTAAGATGGC ATTTTATTTG TCTCATCAGA GGCTCCTGGA TGAAAAGTG	3000
	GATGTGATTA TCTTGATATT CTTGAAAAG CCTCTTCAGA AGTCTAAGTT TCTTCAGCTC	3060
10	AGGAAGAGAC TCTGCAGGAG CTCTGCTCTT GAGTGGCCTG CAAATCCACA GGCTCACCCA	3120
	TACTTCTGGC AGTGCCTGAA AAATGCCCTG ACCACAGACA ATCATGTGGC TTATAGTCAA	3180
	ATGTTCAAGG AAACAGTCTA GCTCTCTGAA GAATGTCACC ACCTAGGACA TGCCTTGAAT	3240
	CGA	3243

**Table 10. Coding Region for Murine TLR7 (5' to 3'; SEQ ID NO:174)**

	ATGGTGTGTTT CGATGTGGAC ACGGAAGAGA CAAATTTGAA TCTTTTTAAA TATGCTCTTA	60
	GTTTCTAGAG TCTTTGGGTT TCGATGGTTT CCTAAAACCTC TACCTTGTGA AGTTAAAGTA	120
	AATATCCCAG AGGCCCATGT GATCGTGGAC TGCACAGACA AGCATTGAC AGAAATCCCT	180
	GAGGGCATTG CCACTAACAC CACCAATCTT ACCCTTACCA TCAACCACAT ACCAACGATC	240
20	TCTCCAGATT CCTTCCGTAG GCTGAACCCT CTGGAAGAAA TCGATTTAAG ATGCAATTGT	300
	GTACCTGTTC TACTGGGGTC CAAAGCCAAT GTGTGTACCA AGAGGCTGCA GATTAGACCT	360
	GGAAGCTTTA GTGGACTCTC TGACTTAAAA GCCCTTACCG TGGATGGAAA CCAACTCTG	420
	GAGATACCAC AGGATCTGCC ATCCAGCTTA CATCTTCTGA GCCTTGAGGC TAACAAACATC	480
	TTCTCCATCA CGAAGGAGAA TCTAACAGAA CTGGTCAACA TTGAAACACT CTACCTGGGT	540
25	CAAAACTGTT ATTATCGAAA TCCTTGCAAT GTTTCCTATT CTATTGAAAA AGATGCTTTC	600
	CTAGTTATGA GAAATTGAA GGTTCTCTCA CTAAAAGATA ACAATGTCAC AGCTGTCCCC	660
	ACCACTTGC CACCTAATT ACTAGAGCTC TATCTTTATA ACAATATCAT TAAGAAAATC	720
	CAAGAAAATG ATTTAATAA CCTCAATGAG TTGCAAGTTC TTGACCTAAG TGGAAATTGC	780
	CCTCGATGTT ATAATGCTCC ATATCCGTGT ACACCGTGT AAAATAATTC CCCCTTACAG	840
30	ATCCATGACA ATGCTTTCAA TTCATTGACA GAATTAAAAG TTTTACGTTT ACACAGTAAT	900
	TCTCTTCAGC ATGTGCCCCC AACATGGTTT AAAACATGA GAAACCTCCA GGAACCTAGAC	960
	CTCTCCAAA ACTACTGGC CAGAGAAATT GAGGAGGCC AATTTTGCA TTTTCTTCCC	1020
	AACCTTGTG AGTTGGATT TTCTTCAAT TATGAGCTGC AGGTCTACCA TGCATCTATA	1080
	ACTTTACAC ATTCACTCTC TTCATTGGAA AACTTGAAAA TTCTGCGTGT CAAGGGTAT	1140
35	GTCTTAAAG AGCTGAAAAA CTCCAGTCTT TCTGTATTGC ACAAGCTTCC CAGGCTGGAA	1200
	GTTCTTGACC TTGGCACTAA CTTCATAAAA ATTGCTGACC TCAACATATT CAAACATTTT	1260
	GAAAACCTCA AACTCATAGA CCTTTCAGTG AATAAGATAT CTCCTTCAGA AGAGTCAAGA	1320
	GAAGTTGGCT TTTGTCTAA TGCTCAAAC TCTGTAGACC GTCACTGGCC CCAGGTCCTT	1380
	GAGGCCTTAC ACTATTTCCG ATACGATGAA TATGCACGGA GCTGCAGGTT CAAAAACAAA	1440
40	GAGCCACCTT CTTTCTTGCC TTTGAATGCA GACTGCCACA TATATGGGCA GACCTTAGAC	1500
	TTAAGTAGAA ATAACATATT TTTTATTAAA CCTTCTGATT TTCAGCATCT TTCATTCCCTC	1560

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	AAATGCCTCA ACTTATCAGG AAACACCATT GGCCAAACTC TTAATGGCAG TGAACCTCTGG	1620
	CCGTTGAGAG AGTTGCGGTA CTTAGACTTC TCCAACAACC GGCTTGATTT ACTCTACTCA	1680
	ACAGCCTTG AAGAGCTCCA GAGTCTTGAA GTTCTGGATC TAAGTAGTAA CAGCCACTAT	1740
5	TTTCAAGCAG AAGGAATTAC TCACATGCTA AACTTTACCA AGAAATTACG GCTTCTGGAC	1800
	AAACTCATGA TGAATGATAA TGACATCTCT ACTTCGGCCA GCAGGACCAT GGAAAGTGAC	1860
	TCTCTTCGAA TTCTGGAGTT CAGAGGCAAC CATTTAGATG TTCTATGGAG AGCCGGTGAT	1920
	AACAGATACT TGGACTTCTT CAAGAATTG TTCAATTAG AGGTATTAGA TATCTCCAGA	1980
10	AATTCCCTGA ATTCCTTGCC TCCTGAGGTT TTTGAGGTA TGCCGCCAAA TCTAAAGAAT	2040
	CTCTCCTTGG CCAAAAATGG GCTCAAATCT TTCTTTGGG ACAGACTCCA GTTACTGAAG	2100
	CATTTGGAAA TTTTGGACCT CAGCCATAAC CAGCTGACAA AAGTACCTGA GAGATTGGCC	2160
	AACTGTTCCA AAAGTCTCAC AACACTGATT CTTAAGCATA ATCAAATCAG GCAATTGACA	2220
	AAATATTTTC TAGAAGATGC TTTGCAATTG CGCTATCTAG ACATCAGTTC AAATAAAATC	2280
	CAGGTCAATTC AGAAGACTAG CTTCCCAGAA AATGTCCTCA ACAATCTGGA GATGTTGGTT	2340
15	TTACATCACA ATCGCTTTCT TTGCAACTGT GATGCTGTGT GGTTGTCTG GTGGGTTAAC	2400
	CATACAGATG TTACTATTCC ATACCTGGCC ACTGATGTGA CTTGTGTTAGG TCCAGGAGCA	2460
	CACAAAGGTC AAAGTGTCAAT ATCCCTTGAT CTGTATACGT GTGAGTTAGA TCTCACAAAC	2520
	CTGATTCTGT TCTCAGTTTC CATATCATCA GTCCTCTTC TTATGGTAGT TATGACAACA	2580
	AGTCACCTCT TTTTCTGGGA TATGTGGTAC ATTTATTATT TTTGGAAAGC AAAGATAAAAG	2640
	GGGTATCAGC ATCTGCAATC CATGGAGTCT TGTTATGATG CTTTTATTGT GTATGACACT	2700
20	AAAAACTCAG CTGTGACAGA ATGGGTTTG CAGGAGCTGG TGGAAAATT GGAAGATCCA	2760
	AGAGAAAAAC ACTTCAATTG GTGTCTAGAA GAAAGAGACT GGCTACCAGG ACAGCCAGTT	2820
	CTAGAAAACC TTTCCCAGAG CATACAGCTC AGCAAAAGA CAGTGTGTGT GATGACACAG	2880
	AAATATGCTA AGACTGAGAG TTTAAGATG GCATTTTATT TGTCTCATCA GAGGCTCCTG	2940
	GATGAAAAG TGGATGTGAT TATCTTGATA TTCTTGAAA AGCCTCTTC GAAAGTCTAAG	3000
25	TTTCTTCAGC TCAGGAAGAG ACTCTGCAGG AGCTCTGTCC TTGAGTGGCC TGCAAATCCA	3060
	CAGGCTCACC CATACTCTG GCAGTGCCTG AAAATGCCG TGACCACAGA CAATCATGTG	3120
	GCTTATAGTC AAATGTTCAA GGAAACAGTC	3150

**Table 11. Amino Acid Sequences of Murine TLR7 and Human TLR7**

30	.	:	.	:	.	:	.	:	.	:	.	60
	hTLR7.pep	MVFPMWTLKRQILILFNIILISKLLGARWFPKTLPCDVTLDPKVNHIVDCTDKHLTEIP	60									
	mTLR7.pep	MVFSMWTRKRQILIFLNMLLVSRVFGFRWFPKTLPCEVKVNIPEAHIVDCTDKHLTEIP	60									
	.	:	.	:	.	:	.	:	.	:	.	120
35	hTLR7.pep	GGIPTNTTNLTLTINHIPDISPASFHRLDHLVEIDFRCNCVPPIPLGSKNMCIKRLQIKP	120									
	mTLR7.pep	EGIPTNTTNLTLTINHIPSISPDSFRRNLHLEEIDLRCNCVPVLLGSKANVCTKRLQIRP	120									
	.	:	.	:	.	:	.	:	.	:	.	180
	hTLR7.pep	RSFSGLTYLKSLYLDGNQLEIPQGLPPSLQLLSLEANNIFSIRKENLTELANIEILYLG	180									
40	mTLR7.pep	GSFSGLSDLKALYLDGNQLEIPQDLPSSLHLLSLEANNIFSITKENLTELNVNIETLYLG	180									
	.	:	.	:	.	:	.	:	.	:	.	240
	hTLR7.pep	QNCYYRNPCYVSYSSIEKDAFLNLTKLVLSKDNNVTAVPTVLPSLTELVLYNNMIAKI	240									

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	mTLR7.pep	QNCYYRNPCNVSYSIEKDAFLVMRNLKVSLKDNNVTAVPTLPPNLLELYLYNNI IKKI	240
	hTLR7.pep	QEDDFNNLNQLQILDLSGNCPRCYNAPFPCAPCKNSPLQIPVNAFDALTELKVRLHSN	300
5	mTLR7.pep	QENDFNNLNELOVLDLSGNCPRCYNVPYPCTPCENNNSPLQIHDNAFNSLTELKVRLHSN	300
	hTLR7.pep	SLQHVPPRWFKNINKLQELDLSQNFLAKEIGDAKFLHFLPSLIQLDLSFNFELQVYRASM	360
	mTLR7.pep	SLQHVPPTWFKNMRNLQELDLSQNYLAREIEEAKFLHFLPNLVELDFSFNYELQVYHASI	360
10			
	hTLR7.pep	NLSQAFSSLKSLKILRIRGYVFKELKSFNLSPHLHNQLQNLEVLDLGTNF KIANLSMFQF	420
	mTLR7.pep	TLPHSLSLENLKILRVKGYVFKELKNSSLSVLHKLPRLEVLDLGTNF KIADLNIFKHF	420
15			
	hTLR7.pep	KRLKVIDLSVNKISPAGDSSEVGFCASNARTSVESYEPQVLEQLHYFRYDKYARSCRFKNK	480
	mTLR7.pep	ENLKLIDLSVNKISPSEESREVGFCPNAQTSDRHPQVLEALHYFRYDEYARSCRFKNK	480
20			
	hTLR7.pep	EA-SFMSVNESCYKGQTLDLSKNSIFFVKSSDFQHLSFLKCLNLSGNLISQTLNGSEFQ	539
	mTLR7.pep	EPPSFLPLNADCHIYGQTLDLSRNNIFFIKPSDFQHLSFLKCLNLSGNTIGQTTLNGSELW	540
25			
	hTLR7.pep	PLAELRYLDFSNRRLDLLHSTAFEELHKLEVLDI SSNSHYFQSEGITHMLNFTKNLKVQ	599
	mTLR7.pep	PLRERLYLDFSNRRLDLLYSTAFEELQSLEVLDLSSNSHYFQAEGITHMLNFTKKLRLLD	600
30			
	hTLR7.pep	KLMMNDNDISSTSRTMESES LRTLEFRGNHLDVLWREGDNRYLQLFKNLLKLEELDISK	660
	mTLR7.pep	KLMMNDNDIST SASRTMESDSL RILEFRGNHLDVLWAGDNRYLDFFKNL FNLEVLDISR	659
	hTLR7.pep	NSLSFLPSGVFDGMPPNLKNLSLAKNGLKSFSWKKLQCLKNLETLDLSHNQLTTVPERLS	719
	mTLR7.pep	NSLNSLPPEV FEGMPPNLKNLSLAKNGLKSFFWDRLQLLKHLEILDLSHNQLTKVPERLA	720
35			
	hTLR7.pep	NCSRSLKNLILKNNQI RSLTKYFLQDAFQLRYLDLSSNKIQMIQKTSFPENVLNNLKMLL	780
	mTLR7.pep	NCSKSLTTLILKHQNQI RQLTKYFLEDALQLRYLDI SSNKIQVIQKTSFPENVLNNLEM LV	779
40			
	hTLR7.pep	LHHNRFLCTCDAVWFVWWVNHTEVTI PYLATDVTCVGPAGKGQSVISLDLYTCELDLTN	840
	mTLR7.pep	LHHNRFLCNCDAVWFVWWVNHTDVTI PYLATDVTCVGPAGKGQSVISLDLYTCELDLTN	839
45			
	hTLR7.pep	LILFSLSISVSLFLMVMMTASHLYFWDVWYIYHFCKAKIKGYQRLISPDCCYDAFI VYDT	900
	mTLR7.pep	LILFSVSISSVLFMVMVMTSHLFFWDMWYIYFWKAKIKGYQHLOSMESCYDAFI VYDT	899

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5	hTLR7.pep	KDPAVTEWVLAELVAKLEDPREKHFNLCLEERDWLPGQPVLLENLSQSIIQLSKKTVFVMTD	959
	mTLR7.pep	KNSAVTEWVLQELVAKLEDPREKHFNLCLEERDWLPGQPVLLENLSQSIIQLSKKTVFVMTQ	960
	bb210788.pep	VDVITILIFLVKPFQKFNFL*LRKRISRSSVLECPPNP	1020
	aa276879.pep	QKSFKFLQLRKRLCRSSVLEWPANP	37
	aa266744.pep	LGKPLQSKFKLQLRKRLCRSSVLEWPANP	24
10	bb116163.pep	IETFQMPSFLSTQRILLDDKVDTVIIILIFLE*PL*KSKFKLQLRKRLFCRSSVLEWPANP	29
	hTLR7.pep	KYAKTENFKIAFYLSHQRLMDEKVDVIIILIFLEKPFQSKFKLQLRKRLCGSSVLEWPANP	56
	mTLR7.pep	KYAKTESFKMAFYLSHQRLLDEKVDVIIILIFLEKPLQSKFKLQLRKRLCRSSVLEWPANP	1019
	bb210788.pep	QAHPYFCQCLKNALTTDNHVAYSQMFKETV	1020
15	aa276879.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1080
	aa266744.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	67
	bb116163.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	54
	hTLR7.pep	QAHPYFWQCLKNALATDNHVAYSQVFKETV	59
	mTLR7.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	86
	bb210788.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1049
	aa276879.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1050
	aa266744.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1050
	bb116163.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1050
	hTLR7.pep	QAHPYFWQCLKNALATDNHVAYSQVFKETV	1050
	mTLR7.pep	QAHPYFWQCLKNALTTDNHVAYSQMFKETV	1050

In Table 11 the sequences are assigned as follows: mTLR7.pep, SEQ ID NO:175; hTLR7.pep, SEQ ID NO:170; bb210788.pep, SEQ ID NO:176; aa276879.pep, SEQ ID NO:177; aa266744.pep, SEQ ID NO:178; and bb116163.pep, SEQ ID NO:179.

### 25 Example 18. Method of cloning human TLR8

Two accession numbers in the GenBank database, AF245703 and AF246971, describe the DNA sequence for human TLR8. To create an expression vector for human TLR8, human TLR8 cDNA was amplified from a cDNA made from human peripheral mononuclear blood cells (PBMC) using the primers 5'-CTGCGCTGCTGCAAGTTACGGAATG-3' (SEQ ID NO:180) and 5'-GCGCGAAATCATGACTAACGTCAG-3 (SEQ ID NO:181). The fragment was cloned into pGEM-T Easy vector (Promega), cut with the restriction enzyme NotI and ligated into a NotI-digested pCDNA3.1 expression vector (Invitrogen). The insert was fully sequenced and translated into protein. The cDNA sequence for hTLR8 is SEQ ID NO:182, is presented in Table 12. The open reading frame starts at base 83, ends at base 3208, and codes for a protein of 1041 amino acids. SEQ ID NO:183 (Table 13), corresponding to bases 83-3205 of SEQ ID NO:182 (Table 12), is the coding region for the polypeptide of SEQ ID NO:184 (Table 14).

The protein sequence of the cloned hTLR8 cDNA matches the sequence described

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under the GenBank accession number AF245703. The sequence deposited under GenBank accession number AF246971 contains an insertion at the N-terminus of 15 amino acids (MKESSLQNSSCSLGKETKK; SEQ ID NO:185) and three single amino acid changes at positions 217 (P to S), 266 (L to P) and 867 (V to I).

5

**Table 12. cDNA Sequence for Human TLR8 (5' to 3'; SEQ ID NO:182)**

	gctccggcc gccatggcgg ccgcggaaat tcgattctgc gctgctgcaa gttacggaat	60
	aaaaaaattag aacaacagaaa acatggaaaa catgttccctt cagtcgtcaa tgctgacctg	120
	cattttcctg ctaatatctg gttccctgtga gttatgcgcc gaagaaaaatt tttctagaag	180
10	ctatccttgt gatgagaaaa agcaaaaatga ctcagttatt gcagagtgcgc gcaatcgctg	240
	actacaggaa gttccccaaa cggtggccaa atatgtgaca gaactagacc tgtctgataa	300
	tttcatcaca cacataacga atgaatcatt tcaagggctg caaaatctca ctaaaaataaa	360
	tctaaaccac aaccccaatg tacagcacca gaacggaaat cccggataac aatcaaatgg	420
	cttgaatatac acagacgggg cattcctcaa cctaaaaaaaaac ctaagggagt tactgcttga	480
15	agacaaccag ttaccccaa taccctctgg tttgccagag tctttgacag aacttagtct	540
	aattcaaaac aatataataca acataactaa agagggcatt tcaagactta taaacttgaa	600
	aatatcttat ttggcctgga actgctattt taacaaagtt tgcgagaaaa ctaacataga	660
	agatggagta tttgaaacgc tgacaaaattt ggagttgcta tcactatctt tcaattctct	720
	ttcacacgtg ccacccaaac tgccaagctc cctacgcaaa cttttctga gcaacaccca	780
20	gatcaaatac attagtgaag aagatttcaa gggattgata aatttaacat tactagattt	840
	aagcggaaac tgtccgaggt gcttcaatgc cccatttcca tgcgtgcctt gtgatggtgg	900
	tgcttcaatt aatataagatc gttttgcttt tcaaaaacttg acccaacttc gataccataaa	960
	cctctcttagc acttccctca ggaagattaa tgctgcctgg tttaaaaata tgcctcatct	1020
	gaaggtgctg gatcttgaat tcaactattt agtgggagaa atagcctctg gggcattttt	1080
25	aacgatgctg ccccgcttag aaatacttga cttgtctttt aactatataa aggggagttt	1140
	tccacagcat attaatattt ccagaaaactt ctctaaactt ttgtctctac gggcattgca	1200
	tttaagaggt tatgtgttcc aggaactcag agaagatgat ttccagcccc tgcagact	1260
	tccaaactta tcgactatca acttgggtat taattttatt aagcaaatcg atttcaaact	1320
	tttccaaaat ttctccaatc tggaaattat ttacttgtca gaaaacagaa tatcaccgtt	1380
30	ggtaaaagat acccggcaga gttatgc当地 tagttccctt ttcaacgtc atatccggaa	1440
	acgacgctca acagattttg agtttgc当地 acattcgaac ttttatttattt tcacccgtcc	1500
	tttaataaaag ccacaatgtg ctgcttatgg aaaagcctt gatthaagcc tcaacagttt	1560
	tttcttctattt gggccaaacc aatttggaaa tcttctgtac attgcctgtt taaatctgtc	1620
	tgcaaatacg aatgctcaag tgttaagtgg aactgaattt tcagccattt ctcattgtcaa	1680
35	atatttggat ttgacaaaaca atagactaga ctttgc当地 gctagtgc当地 ttactgaattt	1740
	gtccgacttg gaagttctag atctcagctt taattcacac tatttc当地 tagcaggcgt	1800
	aacacatcat ctgaaattt ttcaaaaattt cacaatctt aaagttttaa acttgagccaa	1860
	caacaacatt tatactttaa cagataagta taacctggaa agcaagtccc tggtagaattt	1920
	agttttcagt ggcaatcgcc ttgacattttt gtgaaatgat gatgacaaca ggtatatctc	1980
40	cattttcaaa ggtctcaaga atctgacacg tctggattt tcccttaataa ggctgaagca	2040

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	catcccaaat gaagcattcc ttaattgcc agcgagtctc actgaactac atataaatga	2100
	taatatgtta aagttttta actggacatt actccagcag ttccctcgct tcgagttgt	2160
	tgacttacgt gaaaacaaac tacttttt aactgatagc ctatctgact ttacatcttc	2220
5	ccttcggaca ctgctgctga gtcataacag gatttcccac ctaccctctg gcttcttc	2280
	tgaagtcaagt agtctgaagc acctcgattt aagttccaat ctgctaaaaa caatcaacaa	2340
	atccgcacctt gaaaactaaga ccaccaccaa attatctatg ttggaactac acggaaaccc	2400
	ctttgaatgc acctgtgaca ttggagattt ccgaagatgg atggatgaac atctgaatgt	2460
	caaaaattccc agactggtag atgtcatttg tgccagtctt gggatcaaa gagggaaagag	2520
	tattgtgagt ctggagctaa caacttgtgt ttcagatgtc actgcagtga tattatTTT	2580
10	cttcacgttc ttatcacca ccatggttat gttggctgcc ctggctcacc atttgttttta	2640
	ctgggatgtt tggttatata ataatgtgt tttagctaaag gtaaaaggct acaggctct	2700
	ttccacatcc caaactttct atgatgctta catttcttata gacaccaaag acgcctctgt	2760
	tactgactgg gtgataaatg agctgcgcta ccaccttcaa gagagccgag aaaaaaacgt	2820
	tctcctttgt cttagaggaga gggattggaa cccgggattt gccatcatcg acaacccat	2880
15	gcagagcatc aaccaaagca agaaaaacagt atttggtttta accaaaaaat atgcaaaaag	2940
	ctggaaacttt aaaacagctt ttacttggc ttgcagagg ctaatggatg agaacatgga	3000
	tgtgattata ttatcctgc tggagccagt gttacagcat tctcgttatt tgaggctacg	3060
	gcagcggatc tgaagagct ccatcctcca gtggcctgac aacccgaagg cagaaggctt	3120
	gttttggcaa actctgagaa atgtggtctt gactgaaaat gattcacggt ataacaatat	3180
20	gtatgtcgat tccattaagc aatactaact gacgttaagt catgatttcg cgcaatcaact	3240
	agtgaattcg cggccgcctg caggtcgacc atatggaga gctcccaacg cgttggatgc	3300
	atagctttag	3310

**Table 13. Coding Region for Human TLR8 (5' to 3'; SEQ ID NO:183)**

25	atggaaaaaca ttttccttca gtcgtcaatg etgacactgca ttttcctgtt aatatctgg	60
	tcctgtgagt tatgcgccga agaaaaatttt tctagaagct atccttgta tgagaaaaag	120
	caaaaatgact cagttattgc agagtgcagc aatcgctgac tacaggaagt tccccaaacg	180
	gtgggcaaat atgtgacaga actagacctg tctgataatt tcatacacaca cataacgaat	240
	gaatcatttc aagggtcgca aaatctcaact aaaataaaatc taaaccacaa ccccaatgta	300
30	cagcaccaga acggaaatcc cggatatacaa tcaaattggct tgaatatcac agacggggca	360
	ttcctcaacc taaaaaaacctt aaggagttt ctgcttgaag acaaccaggat accccaaata	420
	ccctctggtt tgccagagtc ttgcacagaa cttagtttata ttcaaaaacaa tatataacaac	480
	ataactaaag agggcatttc aagacttata aacttgaaaa atctctattt ggcctggaac	540
	tgctatttttta acaaaggtttgc gggaaaactt aacatagaag atggaggattt tgaaacgctg	600
35	acaaatttgg agttgtatcc actatcttttcaattctcttt cacacgtgcc accccaaactg	660
	ccaagctccc tacgcaaaact ttttctgagc aacacccaga tcaaatacat tagtgaagaa	720
	gatttcaagg gattgataaa tttaacatta ctatgtttaa gcggaaactg tccgaggtgc	780
	ttcaatgcggc catttccatg cgtgccttgc gatgggtggtg cttcaattaa tatagatgt	840
	tttgcttttc aaaacttgac ccaacttcga tacctaaacc tctctagcac ttccctcagg	900
40	aagattaatg ctgcctgggtt taaaaatatg cctcatctga aggtgctgga tcttgaattc	960
	aactatttag tggagaaat agcctctggg gcatttttaa cgatgctgcc ccgttagaa	1020

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	atacttgact tgtcttttaa ctatataaag gggagttatc cacagcatat taatattcc	1080
	agaaaacttct ctaaactttt gtctctacgg gcattgcatt taagaggta tgtgttccag	1140
	gaactcagag aagatgattt ccagccccgt atgcagttc caaacttac gactatcaac	1200
	ttgggtatta attttattaa gcaaatcgat ttcaaacttt tccaaaattt ctccaatctg	1260
5	gaaatttattt acttgtcaga aaacagaata tcaccgttgg taaaagatac ccggcagagt	1320
	tatgcaaata gttcctctt tcaacgtcat atccggaaac gacgctcaac agattttgag	1380
	tttgaccac attcgaactt ttatcatttc acccgtcctt taataaagcc acaatgtgct	1440
	gcttatggaa aagccttaga tttaagcctc aacagtattt tcttcattgg gccaaaccaa	1500
	tttgaaaaatc ttccctgacat tgcctgttta aatctgtctg caaatagcaa tgctcaagt	1560
10	ttaagtggaa ctgaattttc agccattcct catgtcaa atttggattt gacaaacaat	1620
	agactagact ttgataatgc tagtgctctt actgaattgtt ccgacttgga agttctagat	1680
	ctcagctata attcacacta tttcagaata gcaggcgtaa cacatcatct agaatttattt	1740
	caaaatttca caaatctaaa agttttaaac ttgagccaca acaacattt tactttaaca	1800
	gataagtata acctggaaag caagtccctg gttagaattttag ttttcagtgg caatcgccctt	1860
15	gacattttgtt ggaatgatga tgacaacagg tatatctcca ttttcaaagg tctcaagaat	1920
	ctgacacgtc tggatttatac ccttaatagg ctgaagcaca tcccaaataatga agcattcctt	1980
	aatttgcacag cgagtctcac tgaactacat ataaatgata atatgttaaa gtttttaac	2040
	tggacattac tccagcagtt tcctcgtctc gagttgcttgc acttacgtgg aaacaaacta	2100
	ctcttttaa ctgatagcct atctgacttt acatcttccc ttccggacact gctgctgagt	2160
20	cataacacagga ttcccacact accctctggc tttctttctg aagtcaatgt tctgaagcac	2220
	ctcgatttaa gttccaatct gctaaaaaca atcaacaaat ccgcacttga aactaagacc	2280
	accaccaaata tatctatgtt ggaactacac ggaaacccct ttgaatgcac ctgtgacatt	2340
	ggagatttcc gaagatggat ggtgaacat ctgaatgtca aaattccag actggtagat	2400
	gtcatttgc ccagtcctgg ggtcaaaaga gggaaagagta ttgtgagtct ggagctaaca	2460
25	acttgtgttt cagatgtcac tgcagtgtata ttatTTTCT tcacgttctt tatcaccacc	2520
	atggttatgt tggctgccct ggctcaccat ttgtttact gggatgtttg gtttatatat	2580
	aatgtgtgtt tagctaaggat aaaaggctac aggtctctt ccacatccca aactttctat	2640
	gtgcttaca ttcttatga caccaaagac gcctctgtt ctgactgggt gataaatgag	2700
	ctgcgctacc accttgaaga gagccgagac aaaaacgttc tcctttgtct agaggagagg	2760
30	gattgggacc cgggattggc catcatcgac aacctcatgc agagcatcaa ccaaagcaag	2820
	aaaacagtat ttgttttaac caaaaaatat gcaaaaagct ggaactttaa aacagcttt	2880
	tacttggctt tgcagaggct aatggatgag aacatggatg tgattatatt tatcctgctg	2940
	gagccagtgt tacagcattc tcagtttttgc aggctacggc agccgatctg taagagctcc	3000
	atcctccagt ggctgacaa cccgaaggca gaaggcttgc ttggcaaac tctgagaaat	3060
35	gtggtcttga ctgaaaatga ttccacggat aacaatatgt atgtcgattc cattaagcaa	3120
	tac	3123

**Table 14. Amino Acid Sequence of Human TLR8**

	.	:	.	:	.	:	.	:	.	:	.	:	60	
40	AF245703.pep													42
	hTLR8.pep													42
	AF246971.pep	<u>MKES</u>	<u>SLQNS</u>	<u>SSCSL</u>	<u>GKET</u>	<u>KKENMF</u>	<u>LOSSMLTCI</u>	<u>FLLISG</u>	<u>SCELCAE</u>	<u>ENFSRSYPC</u>	<u>DEKKQN</u>			60

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AF245703.pep	DSVIAECSNRLQEVQTVGKYVTELDLSDNFITHITNESFQGLQNLTKINLNHNPNVQH	102			
hTLR8.pep	DSVIAECSNRLQEVQTVGKYVTELDLSDNFITHITNESFQGLQNLTKINLNHNPNVQH	102			
5 AF246971.pep	DSVIAECSNRLQEVQTVGKYVTELDLSDNFITHITNESFQGLQNLTKINLNHNPNVQH	120			
AF245703.pep	QNGNPGIQSNGLNITDGAFLNKLNRELLLEDNQLPQIPSGLPESLTELSLIQNNIYNIT	180			
hTLR8.pep	QNGNPGIQSNGLNITDGAFLNKLNRELLLEDNQLPQIPSGLPESLTELSLIQNNIYNIT	162			
10 AF246971.pep	QNGNPGIQSNGLNITDGAFLNKLNRELLLEDNQLPQIPSGLPESLTELSLIQNNIYNIT	162			
AF245703.pep	KEGISRLINLKLNLYLAWNCYFNKVEKTNIEDGVFETLTNLELLSLSFNSLSHVPPKLPS	240			
hTLR8.pep	KEGISRLINLKLNLYLAWNCYFNKVEKTNIEDGVFETLTNLELLSLSFNSLSHVPPKLPS	222			
15 AF246971.pep	KEGISRLINLKLNLYLAWNCYFNKVEKTNIEDGVFETLTNLELLSLSFNSLSHVSPKLPS	222			
AF245703.pep	SLRKLFSLNTQIKYISEEDFKGLINLTLLDLSGNCPRCFNAFPFCVPCDGGASINIDRFA	300			
hTLR8.pep	SLRKLFSLNTQIKYISEEDFKGLINLTLLDLSGNCPRCFNAFPFCVPCDGGASINIDRFA	282			
20 AF246971.pep	SLRKLFSLNTQIKYISEEDFKGLINLTLLDLSGNCPRCFNAFPFCVPCDGGASINIDRFA	282			
AF245703.pep	FQNLTQLRYLNLSSTSLSRKINAAWFKNMMPHLKVLDLEFNYLVGEIASGAFLTMLPRLEIL	360			
hTLR8.pep	FQNLTQLRYLNLSSTSLSRKINAAWFKNMMPHLKVLDLEFNYLVGEIASGAFLTMLPRLEIL	342			
25 AF246971.pep	FQNLTQLRYLNLSSTSLSRKINAAWFKNMMPHLKVLDLEFNYLVGEIASGAFLTMLPRLEIL	342			
AF245703.pep	DLSFNYIKGSYPQHINISRNFSKLLSLRALHLRGYVFQELREDDFQPLMQLPNLSTINLG	420			
hTLR8.pep	DLSFNYIKGSYPQHINISRNFSKLLSLRALHLRGYVFQELREDDFQPLMQLPNLSTINLG	402			
30 AF246971.pep	DLSFNYIKGSYPQHINISRNFSKPLSLRALHLRGYVFQELREDDFQPLMQLPNLSTINLG	402			
AF245703.pep	INFIKQIDFKLFQNFSNLEIIYLSERISPLVKDTRQSYANSSSFQRHIRKRRSTDFFED	480			
hTLR8.pep	INFIKQIDFKLFQNFSNLEIIYLSERISPLVKDTRQSYANSSSFQRHIRKRRSTDFFED	462			
35 AF246971.pep	INFIKQIDFKLFQNFSNLEIIYLSERISPLVKDTRQSYANSSSFQRHIRKRRSTDFFED	462			
AF245703.pep	PHSNFYHFTRPLIKPQCAAYGKALDLSSLNSIFFIGPNQFENLPDIACLNLSANSNAQVLS	540			
hTLR8.pep	PHSNFYHFTRPLIKPQCAAYGKALDLSSLNSIFFIGPNQFENLPDIACLNLSANSNAQVLS	522			
40 AF246971.pep	PHSNFYHFTRPLIKPQCAAYGKALDLSSLNSIFFIGPNQFENLPDIACLNLSANSNAQVLS	522			
AF245703.pep	GTEFSAIIPHVKYLDLTNNRLDFDNASALTELDILEVLDLSYNSHYFRIAGVTHHLEFIQN	600			
hTLR8.pep	GTEFSAIIPHVKYLDLTNNRLDFDNASALTELDILEVLDLSYNSHYFRIAGVTHHLEFIQN	582			
45 AF246971.pep	GTEFSAIIPHVKYLDLTNNRLDFDNASALTELDILEVLDLSYNSHYFRIAGVTHHLEFIQN	582			

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In Table 14 the sequences are assigned as follows: hTLR8.pep, SEQ ID NO:184; AF245703.pep, SEQ ID NO:186; and AF246971.pep, SEQ ID NO:187.

#### **Example 19. Method of cloning the murine TLR8**

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Alignment of human TLR8 protein sequence with mouse EST database using tfasta yielded 1 hit with mouse EST sequence bf135656. Two primers were designed that bind to bf135656 sequence for use in a RACE-PCR to amplify 5' and 3' ends of the murine TLR8 cDNA. The library used for the RACE PCR was a mouse spleen marathon-ready cDNA 5 commercially available from Clontech. A 5' fragment with a length of 2900 bp and a 3' fragment with a length of 2900 bp obtained by this method were cloned into Promega pGEM-T Easy vector. After sequencing of the 5' end and 3' end of each fragment, partial sequences of mTLR8 were obtained and allowed the design of primers for amplification of the complete murine TLR8 cDNA.

10 Three independent PCR reactions were set up using a spleen murine cDNA from Clontech as a template with the primers 5'-GAGAGAAACAAACGTTTACCTTC-3' (SEQ ID NO:188) and 5'-GATGGCAGAGTCGTGACTTCCC-3' (SEQ ID NO:189). The resulting amplification products were cloned into pGEM-T Easy vector, fully sequenced, translated into protein, and aligned to the human TLR8 protein sequence (GenBank accession number 15 AF245703). The cDNA sequence for mTLR8 is SEQ ID NO:190, presented in Table 15. The open reading frame of mTLR8 starts at base 59, ends at base 3157, and codes for a protein of 1032 amino acids. SEQ ID NO:191 (Table 16), corresponding to bases 59-3154 of SEQ ID NO:190 (Table 15), is the coding region for the polypeptide of SEQ ID NO:192 (Table 17). To create an expression vector for murine TLR8, cDNA pGEM-T Easy vector 20 with the mTLR8 insert was cut with NotI, the fragment isolated, and ligated into a NotI-digested pCDNA3.1 expression vector (Invitrogen).

**Table 15. cDNA Sequence for Murine TLR8 (5' to 3'; SEQ ID NO:190)**

25	attcagagtt ggatgttaag agagaaaacaa acgttttacc ttcctttgtc tatagaacat	60
	ggaaaacatg ccccctcagt catggattct gacgtgctt tgtctgtgt cctctggAAC	120
	cagtgcacatc ttccataaaag cgaactattc cagaagctat ctttgtgacg agataaggca	180
	caactccctt gtgattgcag aatgcaacca tcgtcaactg catgaagtcc cccaaactat	240
	aggcaagttat gtgacaaaaca tagacttgtc agacaatgcc attacacata taacgaaaga	300
	gtccttcaa aagctgcaaa acctcactaa aatcgatctg aaccacaatg ccaaacaaca	360
30	gcacccaaat gaaaataaaaa atggtatgaa tattacagaa ggggcacttc tcagcctaag	420
	aatctaaaca gtttactgc tggaagacaa ccagttatat actatacctg ctgggttgcc	480
	tgagtctttg aaagaactta gcctaattca aaacaatata tttcaggtaa ctaaaaacaa	540
	cactttggg ctttaggaact tggaaagact ctatgggc tggaaactgct atttaaatg	600
	taatcaaacc tttaaggtag aagatgggc attaaaaat cttatacact tgaaggta	660

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	ctcattatct ttcaataacc ttttctatgt gccccccaaa ctaccaagtt ctctaaggaa	720
	actttttctg agtaatgccs aaatcatgaa catcaactcag gaagacttca aaggactgga	780
	aaatttaaca ttactagatc tgagtggaaa ctgtccaagg tgttacaatg ctccatittcc	840
	ttgcacacct tgcaaggaaa actcatccat ccacatacat cctctggctt ttcaaagtct	900
5	cacccaactt ctctatctaa acctttccag cacttccctc aggacgattc cttctacctg	960
	gtttgaaaat ctgtcaaatc tgaaggaact ccatctgaa ttcaactatt tagtcaaga	1020
	aattgcctcg ggggcatttt taacaaaact acccagttt caaatccttg atttgcctt	1080
	caactttcaa tataaggaat atttacaatt tattaatatt tcctcaaatt tctctaaagct	1140
	tcgttctctc aagaagttgc acttaagagg ctatgtgttc cgagaactta aaaagaagca	1200
10	tttcgagcat ctccagagtc ttccaaactt ggcaaccatc aacttgggca ttaactttat	1260
	tgagaaaaatt gatttcaaag ctttccagaa tttttccaaa ctgcacgttta tctattttatc	1320
	aggaaaatcgc atagcatctg tatttagatgg tacagattat tcctcttggc gaaatcgtct	1380
	tcggaaacct ctctcaacag acgatgatga gtttgcattt cacgtgaatt tttaccatag	1440
	caccaaacct ttaataaaagg cacagtgtac tgcttatggc aaggccttgg atttaagttt	1500
15	gaacaatatt ttcttatttgg gaaaaagcca atttgaaggt tttcaggata tcgcctgtt	1560
	aaatctgtcc ttcaatgccs atactcaagt gttaatggc acagaattct cctccatgcc	1620
	ccacataaa tatttggatt taaccaacaa cagactagac tttgatgata acaatgcctt	1680
	cagtgtttt cacgatctag aagtgcgttgc cctgagccac aatgcacact atttcagtat	1740
	agcaggggta acgcaccgtc taggatttat ccagaactta ataaacctca ggggtttaaa	1800
20	cctgagccac aatggcattt acaccctcac agaggaaagt gagctgaaaa gcatctcact	1860
	gaaagaattt gtttcagtg gaaatcgtct tgaccatttgc tggaatgcaaa atgatggcaa	1920
	atactggtcc attttttaaaa gtctccagaa tttgatacgc ctggacttat catacaataa	1980
	ccttcaacaa atccccaaatg gaggatttgc caatttgcct cagagcctcc aagagtact	2040
	tatcagtggc aacaaattac gtttctttaa ttggacatta ctccagtatt ttccctcacct	2100
25	tcacttgctg gatttacgtt gaaatgagct gtattttctt cccaaatttgc tatctaagtt	2160
	tgcacattcc ctggagacac tgctactgag ccataatcat ttctctcacc taccctctgg	2220
	cttcctctcc gaagccagga atctggtgca cctggatcttca agtttcaaca caataaaagat	2280
	gatcaataaa tcctccctgc aaaccaagat gaaaacgaac ttgtcttattc tggagctaca	2340
	tgggaactat ttgactgca cgtgtgacat aagtgatttt cgaagctggc tagatgaaaa	2400
30	tctgaatatc acaatttccata aattggtaaa tgttatatgt tccaaatcctg gggatcaaaa	2460
	atcaaagagt atcatgagcc tagatctcac gacttgcgttca tcggatacca ctgcagctgt	2520
	cctgttttcc ctcacattcc ttaccacccatc catggttatgc ttggctgctc tggttcacca	2580
	cctgttttac tggatgtttt ggtttatcttca tcacatgtgc tctgctaagt taaaaggctt	2640
	caggacttca tccacatccc aaactttcttca tgatgcttattt atttcttattt acacccaaaga	2700
35	tgcacatctgtt actgactggg taatcaatga actgcgttac caccttgcgg agagtgaaga	2760
	caaaaagtgtt ctcctttgtt tagaggagag ggattggat ccaggattac ccatcattga	2820
	taacctccatg cagagcataa accagagcaa gaaaacaatc tttgttttaa ccaagaaata	2880
	tgcacatctgtt actgactggg taatcaatga actgcgttac caccttgcgg agagtgaaga	2940
	gaacatggat gtgatttattt tcacatcttgc ggaaccagtg ttacagtact cacagtaccc	3000
40	gaggcttcgg cagaggatct gtaagagctc catccctccag tggcccaaca atccccaaagc	3060
	agaaaaacttgc ttgtggcaaa gtctgaaaaaa ttgtggatgttgc actgaaaatg attcacggta	3120
	tgacgatttg tacattgttgc atactagtgta tggaaagtca cgactctgcc	3180

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atcataaaaa cacacagctt ctccttacaa tgaaccgaat 3220

**Table 16. Coding Region for Murine TLR8 (5' to 3'; SEQ ID NO:191)**

5	atggaaaaca tgccccctca gtcatggatt ctgacgtgct tttgtctgt tcgcctcttgg accagtgccttccataaa agcgaactat tccagaagct atccttgcata cgagataagg cacaactccc ttgtgattgc agaatgcac catcgtaac tgcataaagt tccccaaact ataggcaagt atgtgacaaa catagacttg tcagacaatg ccattacaca tataacgaaa gagtcctttc aaaagctgca aaacctcaact aaaatcgatc tgaaccacaa tgccaaacaa cagcacccaa atgaaaataa aaatggatg aatattacag aaggggcact tctcagccta 10 agaaaatctaa cagtttact gctggaagac aaccagttat atactatacc tgctgggttgc cctgagtcctt tgaaagaact tagcctaatt caaaaacaata tatttcaggta aactaaaaac aacacttttgc ggcttaggaa cttgaaaga ctctatttgg gctggaactg ctattttaaa tgtaatcaaa cctttaaggt agaagatggg gcatttaaaa atcttataca cttgaaggta ctctcattat ctttcaataa cctttctat gtgccccca aactaccaag ttctctaagg 15 aaacttttgc tgagtaatgc caaaatcatg aacatcaactc aggaagactt caaaggactg gaaaatttaa cattactaga tctgagtgga aactgtccaa ggtgttacaa tgctccattt ccttgcacac cttgcaaggg aaactcatcc atccacatac atcctctggc ttttcaaagt ctcacccaaac ttctctatct aaacctttcc agcacttccc tcaggacgt tccttctacc tggtttggaaa atctgtcaaa tctgaaggaa ctccatctt aattcaacta ttttagttcaa 20 gaaatttgccct cggggggcatt tttaaacaaaa ctacccagtt tacaatccct tgatttgcc ttcaactttc aatataagga atatttacaa tttattaata tttcctcaaa tttctctaag cttcgttctc tcaagaaggt gcacttaaga ggctatgtgt tccgagaact taaaaagaag catttcgagc atctccagag tcttccaaac ttggcaacca tcaacttggg cattaacttt attgagaaaa ttgatttcaa agctttccag aattttcca aactcgacgt tatctattta 25 tcagggaaatc gcatacgatc tgtatttagat ggtacagatt attcctcttgcgcggaaatcgt cttcggaaac ctctctcaac agacgatgtat gagtttgatc cacacgtgaa ttttaccat agcacccaaac cttaataaaa gccacagtgt actgctttagt gcaaggcctt ggatttaagt ttgaacaata ttttcttattt tggaaaagc caatttgaag gttttcagga tatcgctgc ttaaatctgt ctttcaatgc caatactcaa gtgtttaatg gcacagaatt ctccctccatg 30 cccccacatta aatatttggaa tttaaccaac aacagacttag actttgtatga taacaatgct ttcagtgtatc ttacacgtatc agaagtgttg gacctgagcc acaatgcaca ctatttcgt atagcagggg taacgcaccc tcttaggat atccagaact taataaacct cagggtgtta aacctgagcc acaatggcat ttacaccctc acagaggaaa gtgagctgaa aagcatctca ctgaaagaat tggtttctatc tggaaatcgt ctgttgcatt tggaaatgc aatgtatggc 35 aaatactggt ccattttttaa aagtctccatc aatttgcatac gcctggactt atcatacaat aaccttcaac aaatccaaa tggagcattc ctcaatttgc ctcaatgttgc ccaagagtt cttatcgttgc gtaacaaattt acgtttctt aattggacat tactccagta ttttcttcac cttcacttgc tggatttatac gagaaatgag ctgttgcatt tggaaatgc aatgtatggc tttgcacatt ccctggagac actgtactg agccataatc atttctctca cctaccctt 40 ggcttctctt cogaagccatc gaatctggatc cacctggatc taagttcaa cacaataaaag atgatcaata aatcctccct gcaaaaccaag atgaaaacga acttgcataatc tctggagctt 2220 ggcttctctt cogaagccatc gaatctggatc cacctggatc taagttcaa cacaataaaag atgatcaata aatcctccct gcaaaaccaag atgaaaacga acttgcataatc tctggagctt 2280
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	catgggaact atttgactg cacgtgtgac ataagtgatt ttcgaagctg gctagatgaa	2340
	aatctgaata tcacaattcc taaattggta aatgttatat gttccaatcc tggggatcaa	2400
	aaatcaaaaga gtatcatgag cctagatctc acgacttgc tatcgatcac cactgcagct	2460
	gtcctgttt tcctcacatt cttaccacc tccatggta tgtggctgc tctggttcac	2520
5	cacctgtttt actggatgt ttggtttatac tatacatagt gctctgcataa gttaaaaggc	2580
	tacaggactt catccacatc ccaaacttcc tatgatgctt atatttctta tgacaccaaa	2640
	gatgcacatcg ttactgactg ggtaatcaat gaactgcgc accaccttga agagagtgaa	2700
	gacaaaagtgc tcctccttg tttagaggag agggattggg atccaggatt acccattcatt	2760
	gataacctca tgcagagcat aaaccagagc aagaaaaaca tctttgttt aaccaagaaa	2820
10	tatgccaaga gctggaaact taaaacagact ttctacttgg ccttgcagag gctaatggat	2880
	gagaacatgg atgtgattat ttcatcctc ctggaccag tgtacagta ctcacagttac	2940
	ctgaggcttc ggcagaggat ctgtaagagc tccatcctcc agtggcccaa caatcccaa	3000
	gcagaaaaact tggggca aagtctgaaa aatgtggct tgactgaaaa tgattcacgg	3060
	tatgacgatt tgtacattga ttccatttgg caatac	3096

15

**Table 17. Amino Acid Sequences of Murine TLR8 and Human TLR8**

mTLR8 . pep	MENMPPQSWIITCFCLLSSGTSAIFHKANYRSRSPYCDEIRHNSLIVIAECNHRQLHEVPQT	60
hTLR8 . pep	MENMFLQSSMLTCIFLLISGSCELCAEENFSRSPYCDEKKQNDSVIAECSNRRLQEVPQT	60
20		
mTLR8 . pep	IGKYVTNIDLSDNAITHITKESFQKLQNLTIDLNHNAAKQQH----PNEENKNGMNITEGA	120
hTLR8 . pep	VGKYVTELDLSDNFITHITNESFQGLQNLTKINLNHNPNVQHQNNGNPQIQSNGLNITDGA	116
		120
25		
mTLR8 . pep	LLSLRNLTVLLLEDNQLYTIPAGLPESLKELSLIQNNIFQVTKNNTFGLRNLERLYLGWN	176
hTLR8 . pep	FLNLKNLRELLLEDNQLPQIPSGLPESLTELSLIQNNIYNITKEGISRLINLKNLYLAWN	180
		180
30		
mTLR8 . pep	CYFK--CNQTFKVEDGAFKNLIIHLKVLSLSFNNLFYVPPKLPSSLRKLFLSNAKIMNITQ	234
hTLR8 . pep	CYFNKVCEKT-NIEDGVFETLTNLELLSLSFNSLSHVPPKLPSSLRKLFLSNTQIKYISE	239
		240
35		
mTLR8 . pep	EDFKGLENLTLDDLSGNCPRCYNAPPCTPCKENSSIHIHPLAFQSLTQLLYLNLSSTSL	294
hTLR8 . pep	EDFKGLINLTLDDLSGNCPRCFNAPPCTPCKENSSIHIHPLAFQSLTQLRYLNLSSTSL	299
		300
40		
mTLR8 . pep	RTIPSTWFENLSNLKELHLEFNYLVQEIASGAFLTKLPSLQILDLSFNQYKEYLQFINI	354
hTLR8 . pep	RKINAAWFKNMMPHLKVLDLEFNYLVGEIASGAFLTMLPRLEILDLSFNYIKGSYPQHINI	359
		360
mTLR8 . pep	SSNFSKLRSLKKLHLRGYVFRELKKKHFEHLQSLPNLATINLGINFIEKIDFKAFQNF SK	414
hTLR8 . pep	SRNFSKLLSLRALHLRGYVFQELREDDFQPLMQLPNLSTINLGINFIKQIDFKLFQNF SN	419

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	mTLR8 . pep	LDVIYLSGNRIASVLDGT--DY---SSWRNRLRKPLSTDDDEFDPHVNFYHSTKPLIKPQ	480
	hTLR8 . pep	LEIYLSENRISPLVKDTRQSYANSSSFQRHIRKRRSTDF-EFDPHSNFYHFTRPLIKPQ	469
5			540
	mTLR8 . pep	CTAYGKALDLSSLNNIFIIGKSQFEGFQDIACLNLSFNANTQVFNGTEFSSMPHIKYLDLT	529
	hTLR8 . pep	CAAYGKALDLSSLNSIFFIGPNQFENLPDIACLNLSANSNAQVLSGTEFSAIHPVKYLDLT	538
10			600
	mTLR8 . pep	NNRLDFDDNNAFSDLHDLEVLDLSHNAHYFSIAGVTHRLGFIONLINLRVLNLSHNGIYT	589
	hTLR8 . pep	NNRLDFDNASALTELDILEVLDLSYNSHYFRIAGVTHHLEFIQNFTNLKVLNLSHNNIYT	598
15			660
	mTLR8 . pep	LTEESELKSIISLKELVFSGNRLDHLWNANDGKYWSIFKSLQNLIRLDLSYNNLQQIPNGA	649
	hTLR8 . pep	LTDKYNLESKSLVELVFSGNRLDILWNNDDNRYISIFKGLKNLTRLDSLNRLKHIPNEA	658
20			720
	mTLR8 . pep	FLNLPQSLQELLISGNKLRLFFNWTLQLQYFPHLHLLDLSRNELYFLPNCLSKFAHSLETLL	709
	hTLR8 . pep	FLNLPASLTELHINDNMLKFFNWTLQLQFPRLELLDLRGNKLLFLTDSSLSDFTSSLRTLL	718
25			780
	bf135656 . pep	NHFSHLPSGFLSEARNLVHLDLSFNTIKMINKSSLQTKMKTNLSILELHGNYFDCTC	57
	mTLR8 . pep	LSHNHFSHLPSGFLSEARNLVHLDLSFNTIKMINKSSLQTKMKTNLSILELHGNYFDCTC	769
	hTLR8 . pep	LSHRISHLPSGFLSEVSSLKHLDLSSNLLKTINKSALETKTTKLSMELHGNPFECTC	778
30			840
	bf135656 . pep	DISDFRSWLDELNITIPKLVNVICSNPGDQKSIMSDDLTTCVSDTTAAVLFFLTFLT	117
	mTLR8 . pep	DISDFRSWLDELNITIPKLVNVICSNPGDQKSIMSDDLTTCVSDTTAAVLFFLTFLT	829
	hTLR8 . pep	DIGDFRRWMDEHLNVKIPRLVDVICASPGDQRGKSIVSLELTTCVSDVTAVILFFFIFI	838
35			900
	bf135656 . pep	TSMVMLAALVHHLFYWDVWFYIHMCASAKLKGYRTSSTSQTFYDAYISYDTKDASVTDWVI	177
	mTLR8 . pep	TSMVMLAALVHHLFYWDVWFYIHMCASAKLKGYRTSSTSQTFYDAYISYDTKDASVTDWVI	889
	hTLR8 . pep	TTMVMLAALAHHLFYWDVWFYINVCIAKVKGYRSLSSTSQTFYDAYISYDTKDASVTDWVI	898
40			960
	bf135656 . pep	NELRYHLE	185
	mTLR8 . pep	NELRYHLEESEDKSVLLCLEERDWDPGLIIDNLMQSINQSKTIFVLTCKYAKSWNFKT	949
	hTLR8 . pep	NELRYHLEEESRDKNVLLCLEERDWDPGLIIDNLMQSINQSKTIVFVLTCKYAKSWNFKT	958
45			1020
	mTLR8 . pep	AFYLALQRLMDENMDVIIFILEPVLYQSYQLRLRQICKSSILQWPNNPKAENLFWQSL	1009
	hTLR8 . pep	AFYLALQRLMDENMDVIIFILEPVLYQHSQYQLRLRQICKSSILQWPDPNPKAEGLFWQTL	1018
50			1080
	mTLR8 . pep	KNVVLTENDSRYDDLYIDDSIROQY	1032

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hTLR8.pep RNVVLTENDSRYNNMYVDSIKQY

1041

In Table 17 the sequences are assigned as follows: mTLR8.pep, SEQ ID NO:192; hTLR8.pep, SEQ ID NO:184; and bf135656.pep, SEQ ID NO:193.

5

#### **Example 20. Transient transfectants expressing TLR8 and TLR7**

The cloned human TLR7 and human TLR8 cDNA (our result) were cloned into the expression vector pCDNA3.1(-) from Invitrogen using the NotI site. Utilizing a "gain of function" assay, hTLR7 and hTLR8 expression vectors were transiently expressed in human 293 fibroblasts (ATCC, CRL-1573) using the calcium phosphate method. Activation was monitored by IL-8 production after stimulus with CpG-ODN (2006 or 1668, 2 $\mu$ M) or LPS (100 ng/ml). None of the stimuli used activated 293 cells transfected with either hTLR7 or hTLR8.

15 **Example 21. Screening for TLR9, 8 and 7 modulators**

Human TLR receptors 9, 8 and 7 are expressed differentially among tissues which may be due to differences in promoter structure. Du X et al., *Eur Cytokine Netw* 11:362-71 (2000); Chuang TH et al., *Eur Cytokine Netw* 11:372-8 (2000). For the human Toll-like receptors 9, 8 and 7 the genomic locus has been defined and sequenced. TLR9 is located on chromosome 3 (GenBank accession numbers NT\_005985, AC006252), TLR7 on chromosome X (GenBank accession numbers NT\_011774, AC005859, AC003046) and TLR8 close to TLR7 also on chromosome X (GenBank accession numbers NT\_011774, AC005859). To verify differences in the promoter regions the putative promoter region of each gene are cloned in reporter vectors like pGL2-Basic (Promega, Madison, WI, USA) which contains the luciferase gene (luc) adjacent to a multiple cloning site. After transient transfection of these constructs in various cell lines, different stimuli can be tested for the activation of the inserted promoter region which is detected by luciferase activity. The promoter regions defined by the cloning of mTLR9, mTLR8 and mTLR7 can be utilized in the same manner. Definition of compounds that agonize or antagonize TLR9, 8, or 7 expression can be used to enhance or dampen responses to nucleic acid ligands or to any TLR9, 8 or 7 ligand defined by screening. These constructs can be adapted to high throughput screening after stable transfection similar to the use of TLR9 stable transfectants.

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Each of the foregoing patents, patent applications and references is hereby incorporated by reference. While the invention has been described with respect to certain embodiments, it should be appreciated that many modifications and changes may be made by those of ordinary skill in the art without departing from the spirit of the invention. It is  
5 intended that such modification, changes and equivalents fall within the scope of the following claims.

**Example 22. Method cloning the murine and human extracellular TLR9 domain fused to human IgG1 Fc**

10 Human IgG1 Fc was amplified from human B cell cDNA using the sense and antisense primers 5' TATGGATCCTCTTGACAAAAGTCACACATGC (SEQ ID NO:216) and 5' ATA AAGCTTCATTACCCGGAGACAGGGAGAG (SEQ ID NO:217) and ligated into pCDNA3.1(-) (Invitrogen) after digestion with the restriction endonucleases BamHI and HindIII creating the vector pcDNA-IgGFc. The extracellular domain of human  
15 TLR9 (amino acids 1 to 815) was amplified with the sense and antisense primers 5' TATGAATTCCCACCATGGGTTCTGCCGCAG (SEQ ID NO:218) and 5' ATAGGATCCCCGGGGCACCAAGGCCGCCGCCGCCGGAGAGGGCCTCAT CCAGGC (SEQ ID NO:219). The primers amplify the extracellular domain of human TLR9 and create adjacent to amino acid 815 an additional NotI restriction site, a glycine linker and  
20 thrombin protease recognition site. The translated sequence of this region starting at amino acid 812 is DEALSGGRGGGLVPRGS (SEQ ID NO:220). The fragment was cut with EcoRI and BamHI and cloned into pcDNA-IgGFc, creating the vector coding for the fusion protein of the extracellular domain of human TLR9 fused to the Fc part of human IgG1 (pcDNAhTLR9IgGFc). Expressed extracellular TLR9 protein can be separated from the  
25 IgG1 Fc fragment by digestion with Thrombin (see figure).

The extracellular part of murine TLR9 (amino acids 1 to 816) was amplified with the sense and antisense primers 5' TATATCGGCCGCCACCATGGTCTCCGTCGAAG (SEQ ID NO:221) and 5' TATATCGGCCGCCAGAGAGGACCTCATCCAGGC (SEQ ID NO:222) and cloned into pcDNAhTLR9IgGFc after NotI digestion of PCR fragment and  
30 vector. This procedure exchanged the human extracellular part of TLR9 with the murine counterpart.

**Example 23. Method of expression and purification of the extracellular domain of TLR9 fused to human IgG1 Fc**

Vector DNA coding for the human or murine TLR9 human IgGFc fusion protein was transfected by Ca<sub>2</sub>PO<sub>4</sub> method into 293 fibroblast cells. Transfected cells were selected with 0.7 mg/ml G418 and cloned. Expression of fusion protein was monitored by enzyme-linked immunosorbent assay (ELISA). Cells were lysed in lysis buffer (PBS, 1% Triton X-100) and supernatant was applied to ELISA plates coated with polyclonal antibody against human IgG-Fc. Bound fusion protein was detected by incubation with biotinylated polyclonal antibodies against human IgG-Fc and streptavidin-horseradish peroxidase conjugate.

For purification of the fusion protein cell lysates from 10<sup>9</sup> cells were produced and incubated with Protein A sepharose which binds tightly to human IgG-Fc. Incubation with the protease thrombin releases the soluble extracellular domain of human TLR9. **Figure 27** shows an example of the TLR9 fusion protein visualized by a silver stained SDS-gel. **Figure 27** demonstrates that lysates of transfected cells included a strong band travelling between 100 and 150 kD which was not present either in lysates of mock-transfected cells or in supernatants transfected or mock-transfected cells. The apparent molecular weight of the band decreased following thrombin treatment, consistent with cleavage at the thrombin protease recognition site interposed between the extracellular TLR9 domain and the Fc fragment.

**Example 24. Method of cloning the murine and human extracellular TLR7 and TLR8 domain fused to human IgG1 Fc and its expression in 293 cells**

The extracellular domains of murine TLR7 (amino acids 1 to 837), human TLR7 (amino acids 1 to 836), murine TLR8 (amino acids 1 to 816) and human TLR8 (amino acids 1 to 825) were amplified with the primer pairs

5' TATATCGGCCGCCACCATGGTGTTCGATGTGGACACG (SEQ ID NO:223)

and 5' TATATCGGCCGCCATCTAACTCACACGTATAACAGATC (SEQ ID NO:224);

5' TATATCGGCCGCCACCATGGTGTTCGATGTGGACACTG (SEQ ID NO:225)

30 and 5' TATATCGGCCGCCATCTAACTCACAGGTGTACAGATC (SEQ ID NO:226);

5' TATATCGGCCGCCACCATGGAAAACATGCCCTCAG (SEQ ID NO:227) and

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5' TATATCGGGCCGCCATCCGATACACAAGTCGTGAGATC (SEQ ID NO:228); and  
5' TATATCGGGCCGCCACCATGGAAAACATGTTCCCTTCAGTC (SEQ ID NO:229)  
and 5' TATATCGGGCCGCCATCTGAAACACAAAGTTGTTAGCTC (SEQ ID NO:230),  
respectively. Fragments were cloned into pcDNA-IgGFc after NotI digestion.

5 Vector DNA coding for the extracellular domain of human or murine TLR7 or TLR8 fused to human IgGFc fusion protein was transfected by Ca<sub>2</sub>PO<sub>4</sub> method into 293 fibroblast cells. Transfected cells were selected with 0.7 mg/ml G418 and cloned. Expression of fusion protein was monitored by ELISA. Cells were lysed in lysis buffer (PBS, 1% Triton X-100) and supernatant was applied to ELISA plates coated with polyclonal antibody against human  
10 IgG-Fc. Bound fusion protein was detected by incubation with biotinylated polyclonal antibodies against human IgG-Fc and Streptavidin-horseradish peroxidase conjugate.

**Example 25. Method of antibody production against murine and human TLR9 and characterization of activity**

15 C57/B6 mice were immunized three times by intraperitoneal administration of 20 µg of the extracellular domain of human TLR9 mixed with 10 nmol of the CpG-ODN 1668. B cells taken from immunized mice were fused with a non antibody producing B-cell hybridoma P3XAG8 using standard protocols. Hybridoma supernatants were screened for reactivity in ELISA using murine and human TLR9 fusion proteins. For identification of  
20 positive hybridomas ELISA plates were coated with polyclonal antibody against human IgG-Fc and incubated with lysate containing murine or human TLR9 IgG-Fc fusion protein. Plates were then incubated with individual hybridoma supernatants, and bound TLR9-specific antibodies were detected by incubation with biotinylated polyclonal antibodies against murine IgG and Streptavidin-horseradish peroxidase conjugate.

25 Ten antibodies have been isolated which are of IgG1, IgG2a and IgG2b isotype. They have been tested for reactivity against human and murine TLR9 and their performance in western blotting or intracellular staining. Table 18 shows the names (ID), isotypes, reactivity and performance in western blotting and intracellular staining.

30 All isolated antibodies were readily purified using standard protein A affinity chromatography.

**Table 18. Monclonal Antibodies Raised Against Murine and Human TLR9**

#	ID	Isotype	Reactivity in ELISA		Western Blotting	Intracellular Staining
			mTLR9	hTLR9		
1	1-3A11	G1	YES	YES	YES	NO
2	1-1B1	G1	YES	YES	YES	NO
3	1-2A9	G2a	NO	YES	YES	YES
4	1-3F2	G1	YES	YES	YES	NO
5	2-1E2	G2a	NO	YES	YES	YES
6	1-5G5	G2a	YES	YES	YES	YES
7	1-2F1	G1	YES	YES	YES	NO
8	1-5F12	G2b	NO	YES	NO	NO
9	1-3C9	G2a	NO	YES	YES	YES
10	1-3F5	G2b	NO	YES	NO	NO

**Example 26. Method for Intracellular Staining**

Mock transfected 293 cells and human TLR9 transfected 293 cells were seeded on cover slips and cultured overnight. The following day cells were washed in PBS and fixed with 2% formalin for 10 minutes at room temperature. Cells were permeabilized with 0.2% saponin in PBS and incubated with 2 $\mu$ g/ml anti human TLR9-specific antibody 2-1E2 for 1h. After two wash steps cells were incubated with Alexis488-conjugated goat anti-mouse IgG antibody and TLR9 was visualized utilizing confocal microscopy on a Zeiss LSM510 microscope. Results indicated that cytoplasms of human TLR9 transfected 293 cells, but not mock transfected 293 cells, stained positive for human TLR9.

**Example 27. Method for Western Blotting**

Lysates of 293 cells transfected with murine TLR9, human TLR9 or murine TLR2 IgG1-Fc fusion protein were separated by SDS-PAGE. Proteins were transferred to a nylon membrane utilizing a BioRad semi dry blotter according to the manufacturer's protocol. The membrane was incubated with 2 $\mu$ g/ml of the human TLR9-specific antibody 2-1E2, and human TLR9 was detected by polyclonal goat anti-mouse peroxidase conjugate. Peroxidase activity was monitored with ECL reagent (Amersham) and incubation of the membrane on film (see **Figure 29**).

What is claimed is:

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**Claims**

1. An isolated nucleic acid molecule selected from the group consisting of
  - (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence set forth as SEQ ID NO:1, and which code for a murine TLR9 having an amino acid sequence set forth as SEQ ID NO:3,
    - (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code, and
    - (c) complements of (a) or (b).
2. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule codes for SEQ ID NO:3.
3. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule comprises the nucleotide sequence set forth as SEQ ID NO:1.
4. The isolated nucleic acid molecule of claim 1, wherein the isolated nucleic acid molecule comprises a nucleotide sequence set forth as SEQ ID NO:2.
5. An isolated TLR9 polypeptide or fragment thereof comprising at least one amino acid of murine TLR9 selected from the group consisting of amino acids 2, 3, 4, 6, 7, 18, 19, 22, 38, 44, 55, 58, 61, 62, 63, 65, 67, 71, 80, 84, 87, 88, 91, 101, 106, 109, 117, 122, 123, 134, 136, 140, 143, 146, 147, 157, 160, 161, 167, 168, 171, 185, 186, 188, 189, 191, 199, 213, 217, 220, 227, 231, 236, 245, 266, 269, 270, 271, 272, 273, 274, 278, 281, 285, 297, 298, 301, 305, 308, 311, 322, 323, 325, 326, 328, 332, 335, 346, 348, 353, 355, 358, 361, 362, 365, 367, 370, 372, 380, 381, 382, 386, 389, 392, 394, 397, 409, 412, 413, 415, 416, 419, 430, 432, 434, 435, 438, 439, 443, 444, 446, 447, 448, 450, 451, 452, 454, 455, 459, 460, 463, 465, 466, 468, 469, 470, 472, 473, 474, 475, 478, 488, 489, 494, 495, 498, 503, 508, 510, 523, 531, 539, 540, 543, 547, 549, 561, 563, 565, 576, 577, 579, 580, 587, 590, 591, 594, 595, 597, 599, 601, 603, 610, 611, 613, 616, 619, 632, 633, 640, 643, 645, 648, 650, 657, 658, 660, 667, 670, 672, 675, 679, 689, 697, 700, 703, 705, 706, 711, 715, 716, 718, 720, 723, 724, 726, 729, 731, 735, 737, 743, 749, 750, 751, 752, 754, 755, 759, 760,

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772, 774, 780, 781, 786, 787, 788, 800, 814, 821, 829, 831, 832, 835, 844, 857, 858, 859, 862, 864, 865, 866, 879, 893, 894, 898, 902, 910, 917, and 927 of SEQ ID NO:3, wherein the TLR9 polypeptide or fragment thereof has an amino acid sequence which is identical to a human TLR9 polypeptide or fragment thereof except for the at least one amino acid of murine TLR9.

6. The isolated TLR9 polypeptide or fragment thereof of claim 5, further comprising at least one amino acid of murine TLR9 selected from the group consisting of amino acids 949, 972, 975, 976, 994, 997, 1000, 1003, 1004, 1010, 1011, 1018, 1023, and 1027 of SEQ ID NO:3.
7. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the human TLR9 has an amino acid sequence set forth as SEQ ID NO:6.
8. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the isolated TLR9 polypeptide or fragment thereof has an amino acid sequence selected from the group consisting of SEQ ID NO:3 and fragments of SEQ ID NO:3.
9. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the isolated TLR9 polypeptide or fragment thereof is an extracytoplasmic domain of TLR9.
10. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the isolated TLR9 polypeptide or fragment thereof comprises an MBD motif as set forth as SEQ ID NO:126 or SEQ ID NO:127.
11. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the isolated TLR9 polypeptide or fragment thereof selectively binds to an immunostimulatory nucleic acid (ISNA).
12. The isolated TLR9 polypeptide or fragment thereof of claim 5, wherein the isolated TLR9 polypeptide or fragment thereof selectively binds to a CpG nucleic acid.

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13. An isolated nucleic acid molecule which encodes the isolated TLR9 polypeptide or fragment thereof of claim 5.
14. An expression vector comprising the isolated nucleic acid molecule of claim 1 operably linked to a promoter.
15. A host cell comprising the expression vector of claim 14.
16. The host cell of claim 15, further comprising at least one expression vector selected from the group consisting of:
  - (a) an expression vector comprising a nucleic acid molecule which encodes an isolated TLR7 polypeptide operably linked to a promoter, and
  - (b) an expression vector comprising a nucleic acid molecule which encodes an isolated TLR8 polypeptide operably linked to a promoter.
17. The host cell of claim 15, further comprising a reporter construct capable of interacting with a TIR domain.
18. An expression vector comprising the isolated nucleic acid molecule of claim 13 operably linked to a promoter.
19. A host cell comprising the expression vector of claim 18.
20. The host cell of claim 19, further comprising at least one expression vector selected from the group consisting of:
  - (a) an expression vector comprising a nucleic acid molecule which encodes an isolated TLR7 polypeptide operably linked to a promoter, and
  - (b) an expression vector comprising a nucleic acid molecule which encodes an isolated TLR8 polypeptide operably linked to a promoter.

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21. The host cell of claim 19, further comprising a reporter construct capable of interacting with a TIR domain.
22. An isolated nucleic acid molecule selected from the group consisting of
  - (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence set forth as SEQ ID NO:173, and which code for a murine TLR7 having an amino acid sequence set forth as SEQ ID NO:175,
  - (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code, and
  - (c) complements of (a) or (b).
23. The isolated nucleic acid molecule of claim 22, wherein the isolated nucleic acid molecule codes for SEQ ID NO:175.
24. The isolated nucleic acid molecule of claim 22, wherein the isolated nucleic acid molecule comprises the nucleotide sequence set forth as SEQ ID NO:173.
25. The isolated nucleic acid molecule of claim 22, wherein the isolated nucleic acid molecule comprises a nucleotide sequence set forth as SEQ ID NO:174.
26. An isolated TLR7 polypeptide or fragment thereof comprising at least one amino acid of murine TLR7 selected from the group consisting of amino acids 4, 8, 15, 16, 18, 21, 23, 24, 25, 27, 37, 39, 40, 41, 42, 44, 45, 61, 79, 83, 86, 89, 92, 96, 103, 109, 111, 113, 119, 121, 127, 128, 131, 145, 148, 151, 164, 172, 176, 190, 202, 203, 204, 205, 222, 225, 226, 228, 236, 238, 243, 250, 253, 266, 268, 271, 274, 282, 283, 287, 288, 308, 313, 314, 315, 325, 328, 331, 332, 341, 343, 344, 347, 351, 357, 360, 361, 362, 363, 364, 365, 366, 370, 371, 377, 378, 387, 388, 389, 392, 397, 398, 413, 415, 416, 419, 421, 422, 425, 437, 438, 440, 446, 449, 453, 454, 455, 456, 462, 470, 482, 486, 487, 488, 490, 491, 493, 494, 503, 505, 509, 511, 529, 531, 539, 540, 543, 559, 567, 568, 574, 583, 595, 597, 598, 600, 611, 613, 620, 624, 638, 645, 646, 651, 652, 655, 660, 664, 665, 668, 669, 672, 692, 694, 695, 698, 701, 704, 714, 720, 724, 727, 728, 733, 738, 745, 748, 755, 762, 777, 780, 789,

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803, 846, 850, 851, 860, 864, 868, 873, 875, 884, 886, 888, 889, 890, 902, 903, 911, 960, 967, 970, 980, 996, 1010, 1018, 1035, and 1045 of SEQ ID NO:175, wherein the TLR7 polypeptide or fragment thereof has an amino acid sequence which is identical to a human TLR7 polypeptide or fragment thereof except for the at least one amino acid of murine TLR7.

27. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the human TLR7 has an amino acid sequence set forth as SEQ ID NO:170.
28. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the isolated TLR7 polypeptide or fragment thereof has an amino acid sequence selected from the group consisting of SEQ ID NO:175 and fragments of SEQ ID NO:175.
29. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the isolated TLR7 polypeptide or fragment thereof is an extracytoplasmic domain of TLR7.
30. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the isolated TLR7 polypeptide or fragment thereof comprises an MBD motif as set forth as any one of SEQ ID NOS: 203, 204, 212, and 213.
31. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the isolated TLR7 polypeptide or fragment thereof selectively binds to an ISNA.
32. The isolated TLR7 polypeptide or fragment thereof of claim 26, wherein the isolated TLR7 polypeptide or fragment thereof selectively binds to a CpG nucleic acid.
33. An isolated nucleic acid molecule which encodes the isolated TLR7 polypeptide or fragment thereof of claim 26.
34. An expression vector comprising the isolated nucleic acid molecule of claim 22 operably linked to a promoter.

35. A host cell comprising the expression vector of claim 34.
36. The host cell of claim 35, further comprising a reporter construct capable of interacting with a TIR domain.
37. An expression vector comprising the isolated nucleic acid molecule of claim 33 operably linked to a promoter.
38. A host cell comprising the expression vector of claim 37.
39. The host cell of claim 38, further comprising a reporter construct capable of interacting with a TIR domain.
40. An isolated nucleic acid molecule selected from the group consisting of
  - (a) nucleic acid molecules which hybridize under stringent conditions to a nucleic acid molecule having a nucleotide sequence set forth as SEQ ID NO:190, and which code for a murine TLR8 having an amino acid sequence set forth as SEQ ID NO:192,
  - (b) nucleic acid molecules that differ from the nucleic acid molecules of (a) in codon sequence due to degeneracy of the genetic code, and
  - (c) complements of (a) or (b).
41. The isolated nucleic acid molecule of claim 40, wherein the isolated nucleic acid molecule codes for SEQ ID NO:192.
42. The isolated nucleic acid molecule of claim 40, wherein the isolated nucleic acid molecule comprises the nucleotide sequence set forth as SEQ ID NO:190.
43. The isolated nucleic acid molecule of claim 40, wherein the isolated nucleic acid molecule comprises a nucleotide sequence set forth as SEQ ID NO:191.

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44. An isolated TLR8 polypeptide or fragment thereof comprising at least one amino acid of murine TLR8 selected from the group consisting of amino acids 5, 6, 9, 10, 14, 15, 18, 21, 22, 23, 24, 25, 26, 27, 28, 30, 39, 40, 41, 43, 44, 50, 51, 53, 55, 61, 67, 68, 74, 80, 85, 93, 98, 99, 100, 104, 105, 106, 107, 110, 114, 117, 119, 121, 124, 125, 134, 135, 138, 145, 155, 156, 157, 160, 161, 162, 163, 164, 166, 169, 170, 174, 180, 182, 183, 186, 187, 191, 193, 194, 196, 197, 199, 200, 207, 209, 210, 227, 228, 230, 231, 233, 234, 241, 256, 263, 266, 267, 268, 269, 272, 274, 275, 276, 280, 285, 296, 298, 299, 300, 303, 305, 306, 307, 310, 312, 320, 330, 333, 335, 343, 344, 345, 346, 347, 349, 351, 356, 362, 365, 366, 375, 378, 379, 380, 381, 383, 384, 386, 387, 392, 402, 403, 408, 414, 416, 417, 422, 426, 427, 428, 429, 430, 431, 433, 437, 438, 439, 440, 441, 444, 445, 449, 456, 461, 463, 471, 483, 486, 489, 490, 494, 495, 496, 505, 507, 509, 512, 513, 519, 520, 523, 537, 538, 539, 541, 542, 543, 545, 554, 556, 560, 567, 569, 574, 575, 578, 586, 592, 593, 594, 595, 597, 599, 602, 613, 617, 618, 620, 621, 623, 628, 630, 633, 639, 641, 643, 644, 648, 655, 658, 661, 663, 664, 666, 668, 677, 680, 682, 687, 688, 690, 692, 695, 696, 697, 700, 702, 703, 706, 714, 715, 726, 727, 728, 730, 736, 738, 739, 741, 746, 748, 751, 752, 754, 757, 764, 766, 772, 776, 778, 781, 784, 785, 788, 791, 795, 796, 801, 802, 806, 809, 817, 820, 821, 825, 828, 829, 831, 839, 852, 853, 855, 858, 863, 864, 900, 903, 911, 918, 934, 977, 997, 1003, 1008, 1010, 1022, 1023, 1024, 1026, and 1030 of SEQ ID NO:192, wherein the TLR8 polypeptide or fragment thereof has an amino acid sequence which is identical to a human TLR8 polypeptide or fragment thereof except for the at least one amino acid of murine TLR8.

45. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the human TLR8 has an amino acid sequence set forth as SEQ ID NO:184.

46. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the isolated TLR8 polypeptide or fragment thereof has an amino acid sequence selected from the group consisting of SEQ ID NO:192 and fragments of SEQ ID NO:192.

47. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the isolated TLR8 polypeptide or fragment thereof is an extracytoplasmic domain of TLR8.

48. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the isolated TLR8 polypeptide or fragment thereof comprises an MBD motif as set forth as any one of SEQ ID NOs: 205, 206, 214, and 215.
49. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the isolated TLR8 polypeptide or fragment thereof selectively binds to an ISNA.
50. The isolated TLR8 polypeptide or fragment thereof of claim 44, wherein the isolated TLR8 polypeptide or fragment thereof selectively binds to a CpG nucleic acid.
51. An isolated nucleic acid molecule which encodes the isolated TLR8 polypeptide or fragment thereof of claim 44.
52. An expression vector comprising the isolated nucleic acid molecule of claim 40 operably linked to a promoter.
53. A host cell comprising the expression vector of claim 52.
54. The host cell of claim 53, further comprising a reporter construct capable of interacting with a TIR domain.
55. An expression vector comprising the isolated nucleic acid molecule of claim 51 operably linked to a promoter.
56. A host cell comprising the expression vector of claim 55.
57. The host cell of claim 56, further comprising a reporter construct capable of interacting with a TIR domain.
58. An isolated nucleic acid molecule which hybridizes under stringent conditions to the

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- isolated nucleic acid molecule of claim 1 or claim 13.
59. A method for inhibiting TLR9 signaling activity in a cell, comprising:  
contacting the cell with an isolated nucleic acid molecule of claim 58 in an amount effective to inhibit expression of TLR9 polypeptide in the cell.
60. An isolated nucleic acid molecule comprising a nucleotide sequence which is complementary to the nucleotide sequence of the isolated nucleic acid molecule of claim 1 or claim 13.
61. A method for inhibiting TLR9 signaling activity in a cell, comprising:  
contacting the cell with an isolated nucleic acid molecule of claim 60 in an amount effective to inhibit expression of TLR9 polypeptide in the cell.
62. A method for identifying nucleic acid molecules which interact with a TLR polypeptide or a fragment thereof, comprising:  
contacting a TLR polypeptide selected from the group consisting of TLR7, TLR8, TLR9, and nucleic acid-binding fragments thereof with a test nucleic acid molecule; and measuring an interaction of the test nucleic acid molecule with the TLR polypeptide or fragment thereof.
63. The method of claim 62, wherein the TLR polypeptide or fragment thereof is expressed in a cell.
64. The method of claim 62, wherein the TLR polypeptide or fragment thereof is an isolated TLR polypeptide or fragment thereof.
65. The method of claim 64, wherein the isolated TLR polypeptide or fragment thereof is immobilized on a solid support.
66. The method of claim 62, wherein the TLR polypeptide or fragment thereof is fused

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with an Fc fragment of an antibody.

67. The method of claim 66, wherein the TLR polypeptide or fragment thereof comprises a TLR extracytoplasmic domain.
68. The method of claim 62, wherein the interaction is binding.
69. The method of claim 68, wherein the measuring is accomplished by a method selected from the group consisting of enzyme-linked immunosorbent assay (ELISA), biomolecular interaction assay (BIA), electromobility shift assay (EMSA), radioimmunoassay (RIA), polyacrylamide gel electrophoresis (PAGE), and Western blotting.
70. The method of claim 63, wherein the measuring is accomplished by a method comprising measuring a response mediated by a TLR signal transduction pathway.
71. The method of claim 70, wherein the response mediated by a TLR signal transduction pathway is selected from the group consisting of induction of a gene under control of NF- $\kappa$ B promoter and secretion of a cytokine.
72. The method of claim 71, wherein the gene under control of NF- $\kappa$ B promoter is selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc.
73. The method of claim 71, wherein the cytokine is selected from the group consisting of IL-8, TNF- $\alpha$ , and IL-12 p40.
74. The method of claim 70, further comprising:  
comparing (a) the response mediated by a TLR signal transduction pathway as measured in presence of the test nucleic acid molecule with (b) a response mediated by a TLR signal transduction pathway as measured in absence of the test nucleic acid molecule; and

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determining the test nucleic acid molecule is an ISNA when (a) exceeds (b).

75. The method of claim 70, further comprising:  
comparing the response to a reference response when the TLR polypeptide is independently contacted with a reference nucleic acid molecule; and  
determining if the response is stronger or weaker than the reference response.
76. The method of claim 70, further comprising:  
comparing the response to a reference response when the TLR polypeptide is concurrently contacted with a reference nucleic acid molecule; and  
determining if the response is stronger or weaker than the reference response.
77. The method of claim 62, wherein the TLR polypeptide or fragment thereof is TLR7.
78. The method of claim 62, wherein the TLR polypeptide or fragment thereof is TLR8.
79. The method of claim 62, wherein the TLR polypeptide or fragment thereof is TLR9.
80. A screening method for identifying an ISNA, comprising:  
contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a test nucleic acid molecule;  
detecting presence or absence of a response mediated by a TLR signal transduction pathway in the presence of the test nucleic acid molecule arising as a result of an interaction between the functional TLR and the test nucleic acid molecule; and  
determining the test nucleic acid molecule is an ISNA when the presence of a response mediated by the TLR signal transduction pathway is detected.
81. The method of claim 80, further comprising comparing the response mediated by the TLR signal transduction pathway arising as a result of an interaction between the functional TLR and the test nucleic acid molecule with a response arising as a result of an interaction between the functional TLR and a reference ISNA.

82. The method of claim 81, wherein the screening method is performed on a plurality of test nucleic acid molecules.
83. The method of claim 82, wherein the response mediated by the TLR signal transduction pathway is measured quantitatively and wherein the response mediated by the TLR signal transduction pathway associated with each of the plurality of test nucleic acid molecules is compared with a response arising as a result of an interaction between the functional TLR and a reference ISNA.
84. The method of claim 83, wherein a subset of the plurality of test nucleic acid molecules is selected based on ability of the subset to produce a specific response mediated by the TLR signal transduction pathway.
85. The method of claim 80, wherein the functional TLR is expressed in a cell.
86. The method of claim 85, wherein the cell is an isolated mammalian cell that naturally expresses the functional TLR.
87. The method of claim 86, wherein the cell comprises an expression vector comprising an isolated nucleic acid which encodes a reporter construct selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc.
88. The method of claim 80, wherein the functional TLR is part of a cell-free system.
89. The method of claim 80, wherein the functional TLR is part of a complex with another TLR.
90. The method of claim 89, wherein the complex is a complex of TLR9 and TLR7.
91. The method of claim 89, wherein the complex is a complex of TLR9 and TLR8.

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92. The method of claim 80, wherein the functional TLR is part of a complex with a non-TLR protein selected from the group consisting of MyD88, IRAK, TRAF6, I<sub>K</sub>B, NF-<sub>κ</sub>B, and functional homologues and derivatives thereof.
93. The method of claim 80, wherein the reference ISNA is a CpG nucleic acid.
94. The method of claim 80, wherein the test nucleic acid molecule is a CpG nucleic acid.
95. The method of claim 80, wherein the response mediated by a TLR signal transduction pathway is selected from the group consisting of induction of a gene under control of NF-<sub>κ</sub>B promoter and secretion of a cytokine.
96. The method of claim 95, wherein the gene under control of NF-<sub>κ</sub>B promoter is selected from the group consisting of IL-8, IL-12 p40, NF-<sub>κ</sub>B-luc, IL-12 p40-luc, and TNF-luc.
97. The method of claim 95, wherein the cytokine is selected from the group consisting of IL-8, TNF- $\alpha$ , and IL-12 p40.
98. A screening method for comparing TLR signaling activity of a test compound with an ISNA, comprising:
  - contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a reference ISNA and detecting a reference response mediated by a TLR signal transduction pathway;
  - contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 with a test compound and detecting a test response mediated by a TLR signal transduction pathway; and
  - comparing the test response with the reference response to compare the TLR signaling activity of the test compound with the ISNA.

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99. The method of claim 98, wherein the functional TLR is contacted with the reference ISNA and the test compound independently.

100. The method of claim 99, wherein the screening method is a method for identifying an ISNA mimic, and wherein when the test response is similar to the reference response the test compound is an ISNA mimic.

101. The method of claim 98, wherein the functional TLR is contacted with the reference ISNA and the test compound concurrently to produce a test-reference response mediated by a TLR signal transduction pathway and wherein the test-reference response may be compared to the reference response.

102. The method of claim 101, wherein the screening method is a method for identifying an ISNA agonist, and wherein when the test-reference response is greater than the reference response the test compound is an ISNA agonist.

103. The method of claim 101, wherein the screening method is a method for identifying an ISNA antagonist, and wherein when the test-reference response is less than the reference response the test compound is an ISNA antagonist.

104. The method of claim 98, wherein the functional TLR is expressed in a cell.

105. The method of claim 104, wherein the cell is an isolated mammalian cell that naturally expresses the functional TLR9.

106. The method of claim 105, wherein the cell comprises an expression vector comprising an isolated nucleic acid which encodes a reporter construct selected from the group consisting of IL-8, IL-12 p40, NF- $\kappa$ B-luc, IL-12 p40-luc, and TNF-luc.

107. The method of claim 98, wherein the functional TLR is part of a cell-free system.

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108. The method of claim 98, wherein the functional TLR is part of a complex with another TLR.
109. The method of claim 98, wherein the functional TLR is part of a complex with a non-TLR protein selected from the group consisting of MyD88, IRAK, TRAF6, I $\kappa$ B, NF- $\kappa$ B, and functional homologues and derivatives thereof.
110. The method of claim 98, wherein the reference ISNA is a CpG nucleic acid.
111. The method of claim 98, wherein the test compound is not a nucleic acid molecule.
112. The method of claim 98, wherein the test compound is a polypeptide.
113. The method of claim 98, wherein the test compound is a part of a combinatorial library of compounds.
114. A screening method for identifying species specificity of an ISNA, comprising:
  - contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a first species with a test ISNA;
  - contacting a functional TLR selected from the group consisting of TLR7, TLR8, and TLR9 of a second species with the test ISNA;
  - measuring a response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test ISNA;
  - measuring a response mediated by the TLR signal transduction pathway associated with the contacting the functional TLR of the second species with the test ISNA; and
  - comparing (a) the response mediated by a TLR signal transduction pathway associated with the contacting the functional TLR of the first species with the test ISNA with (b) the response mediated by the TLR signal transduction pathway associated with the contacting the functional TLR of the second species with the test ISNA.
115. The method of claim 114, wherein the functional TLR is expressed in a cell.

116. The method of claim 115, wherein the cell is an isolated mammalian cell that naturally expresses the functional TLR.

117. The method of claim 114, wherein the functional TLR is part of a cell-free system.

118. The method of claim 114, wherein the functional TLR is part of a complex with another TLR.

119. The method of claim 114, wherein the functional TLR is part of a complex with a non-TLR protein selected from the group consisting of MyD88, IRAK, TRAF6, I $\kappa$ B, NF- $\kappa$ B, and functional homologues and derivatives thereof.

120. A method for identifying lead compounds for a pharmacological agent useful in treatment of disease associated with TLR9 signaling activity, comprising providing a cell comprising a TLR9 as provided in claim 5; contacting the cell with a candidate pharmacological agent under conditions which, in the absence of the candidate pharmacological agent, cause a first amount of TLR9 signaling activity; and determining a second amount of TLR9 signaling activity as a measure of the effect of the pharmacological agent on the TLR9 signaling activity, wherein a second amount of TLR9 signaling activity which is less than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which reduces TLR9 signaling activity and wherein a second amount of TLR9 signaling activity which is greater than the first amount indicates that the candidate pharmacological agent is a lead compound for a pharmacological agent which increases TLR9 signaling activity.

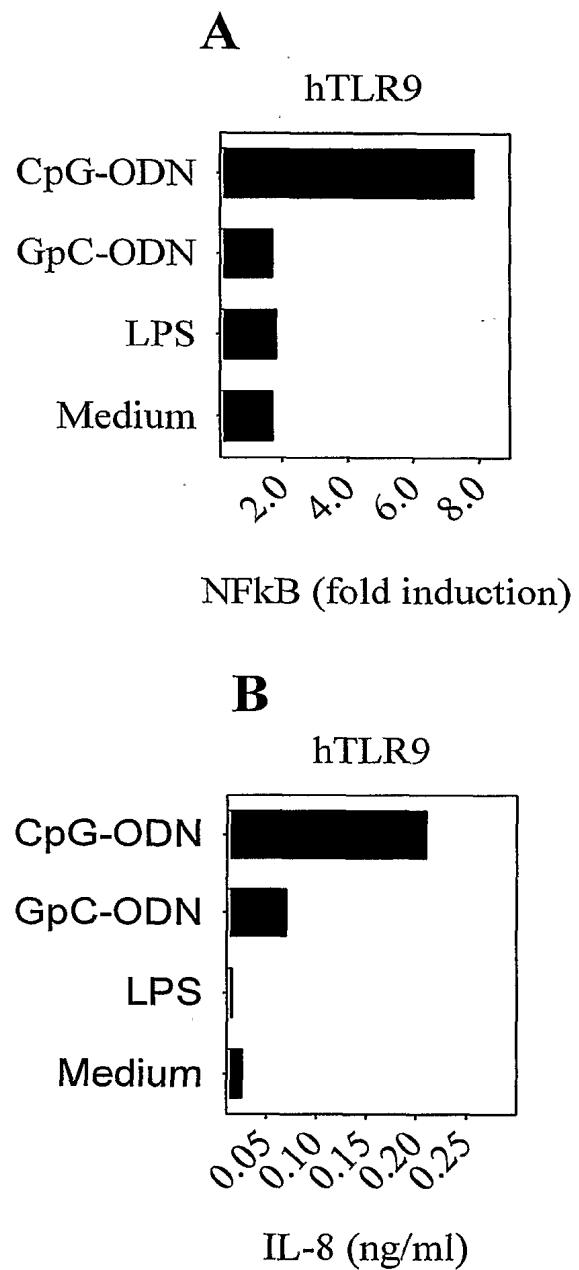


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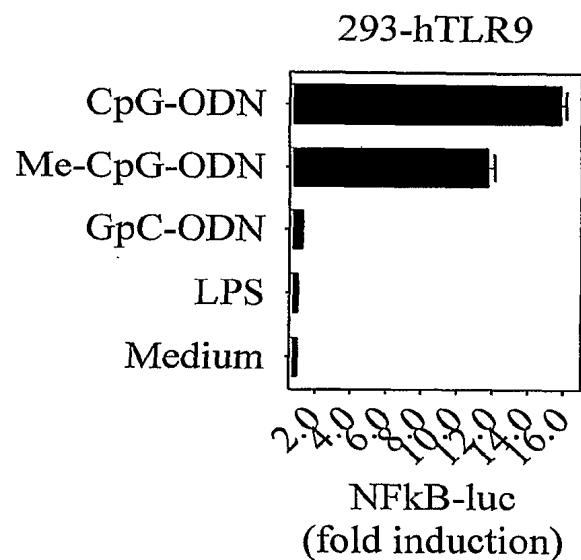


Figure 2

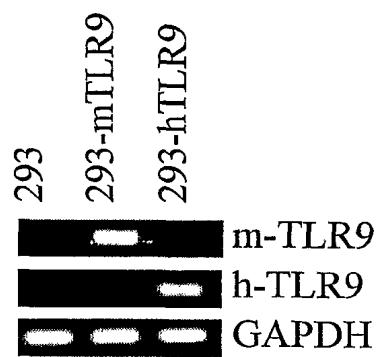


Figure 3

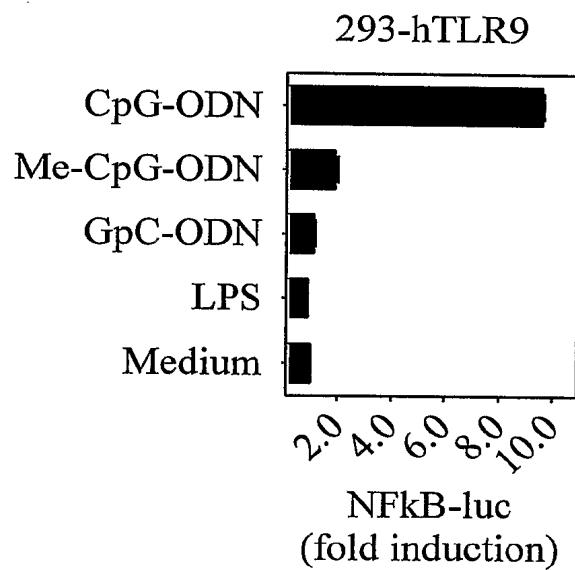


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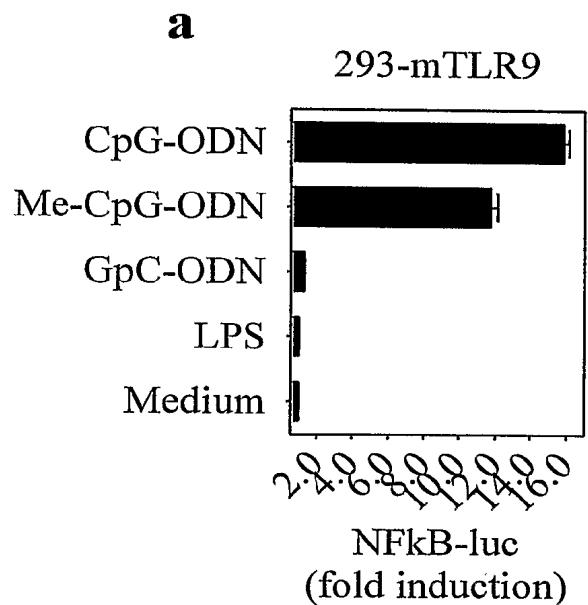


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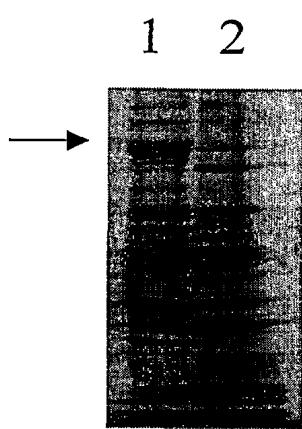


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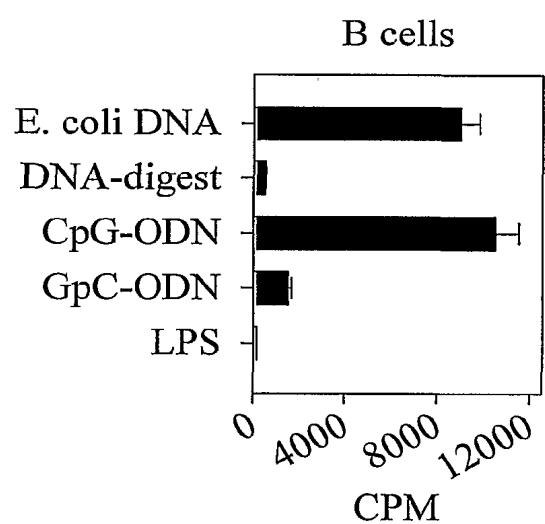


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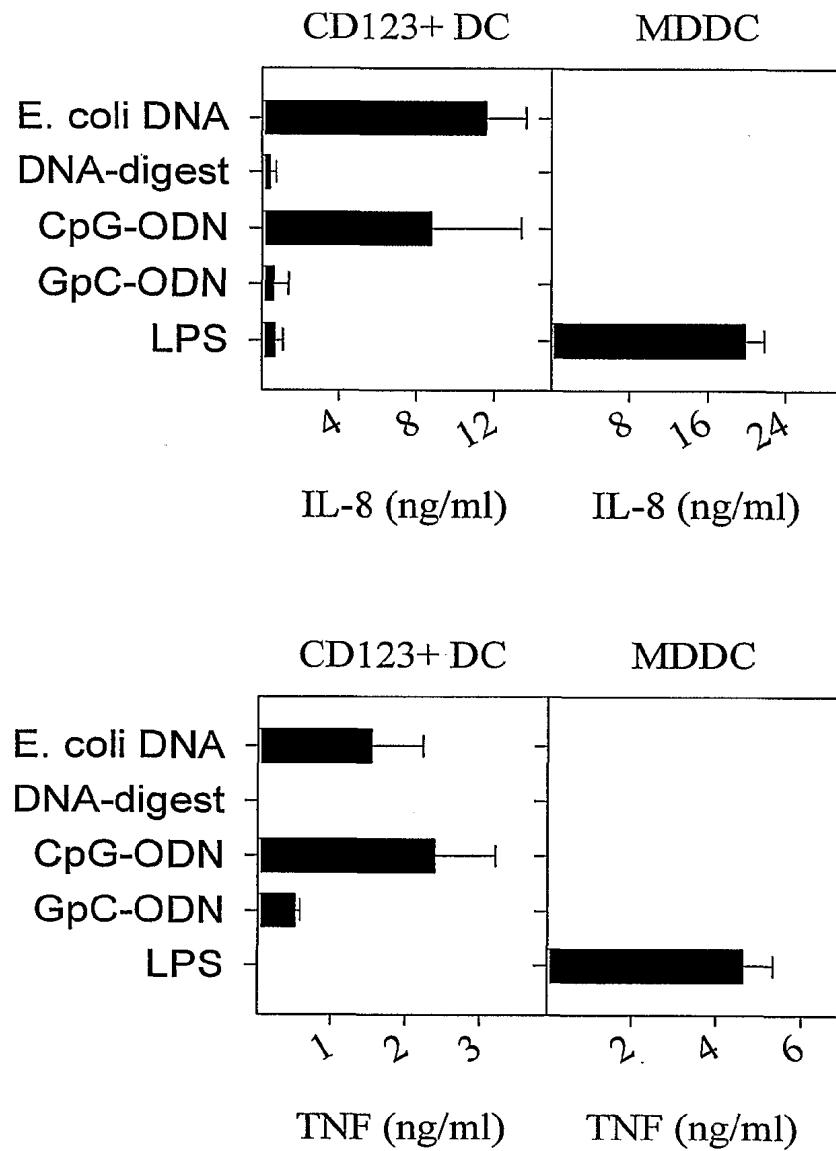


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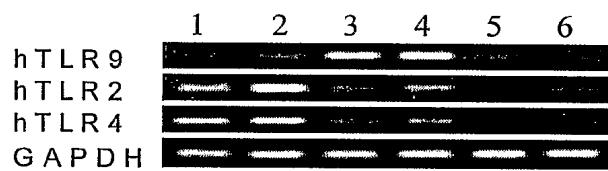


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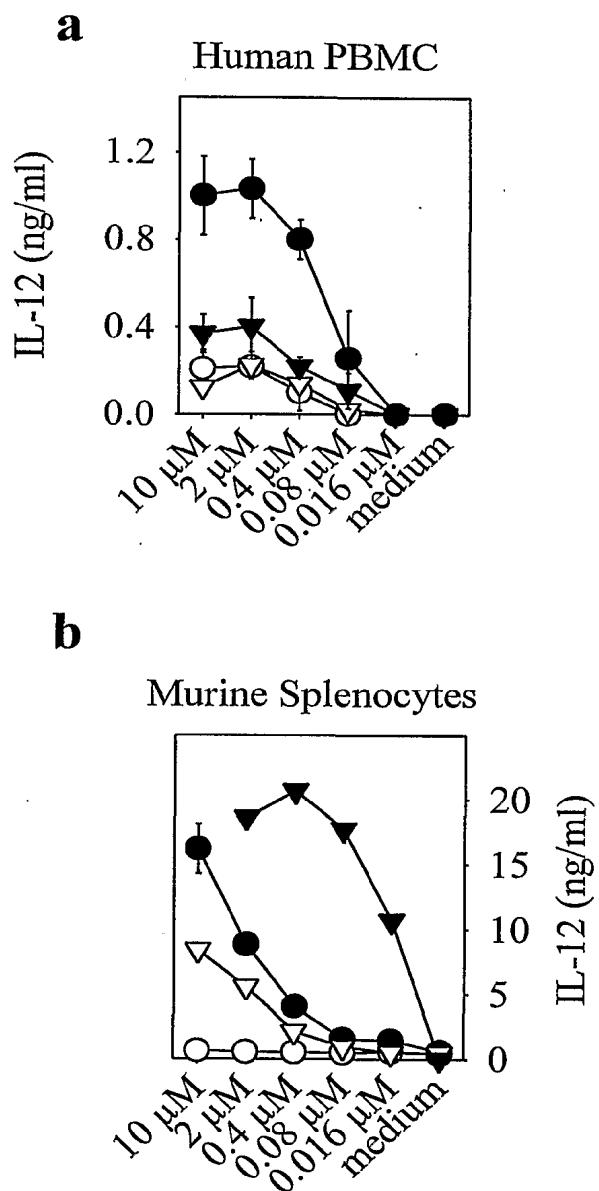


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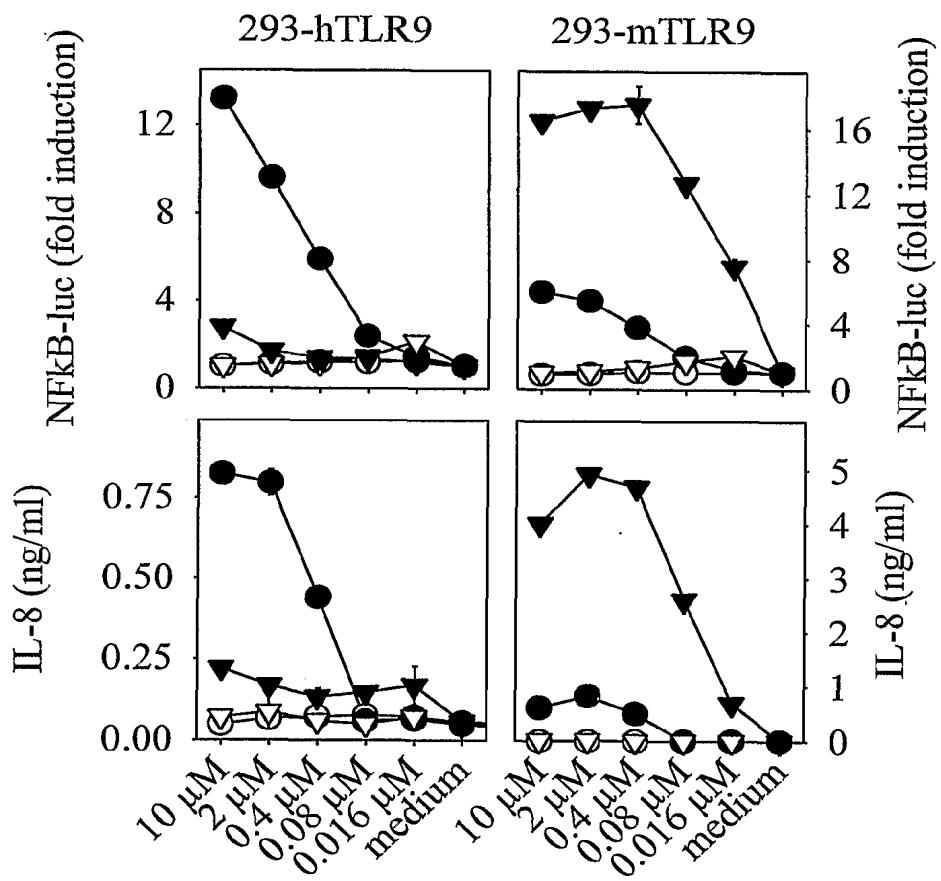


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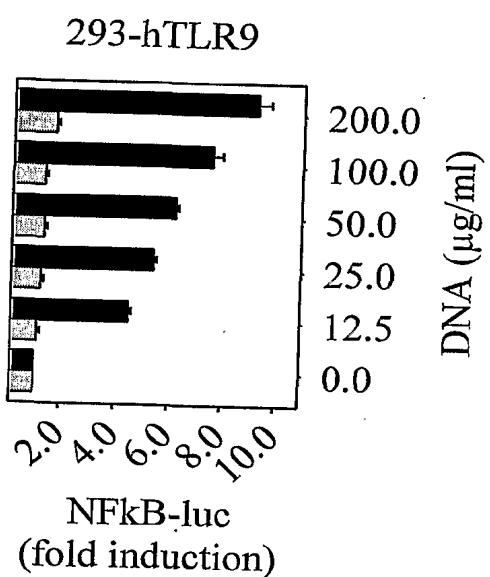
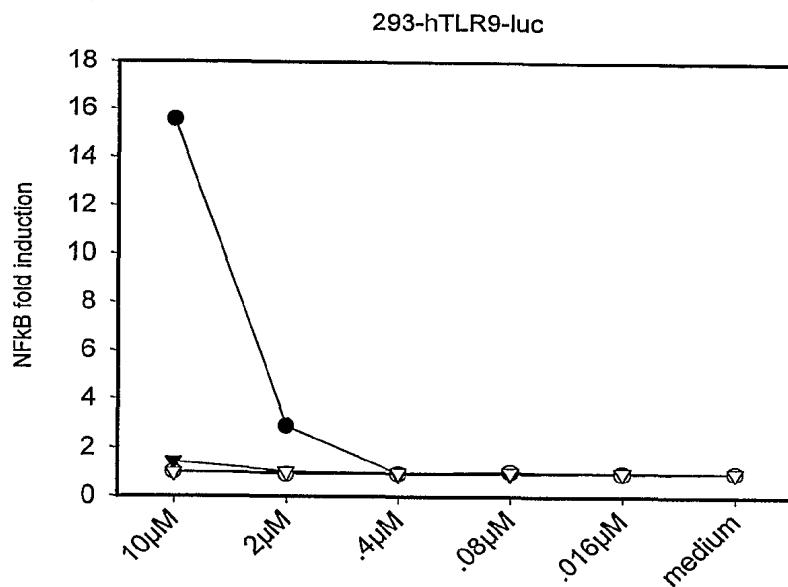
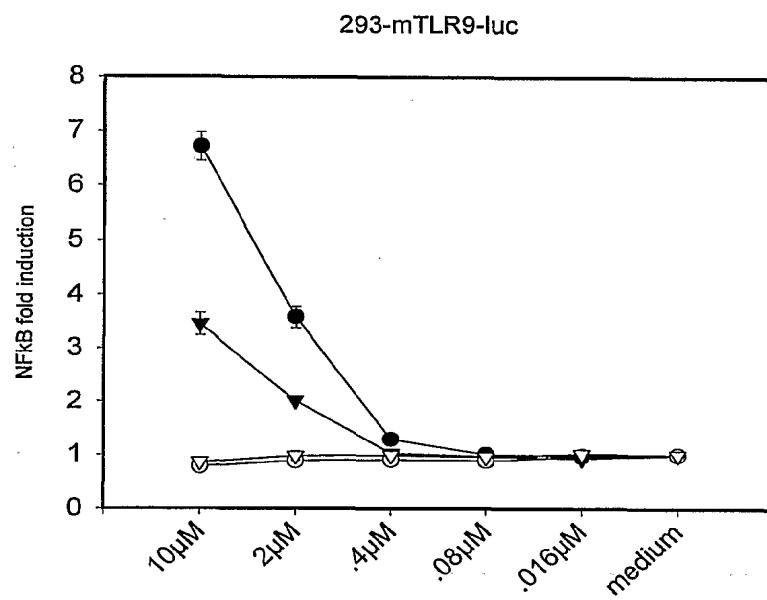


Figure 12

**A****B****Figure 13**

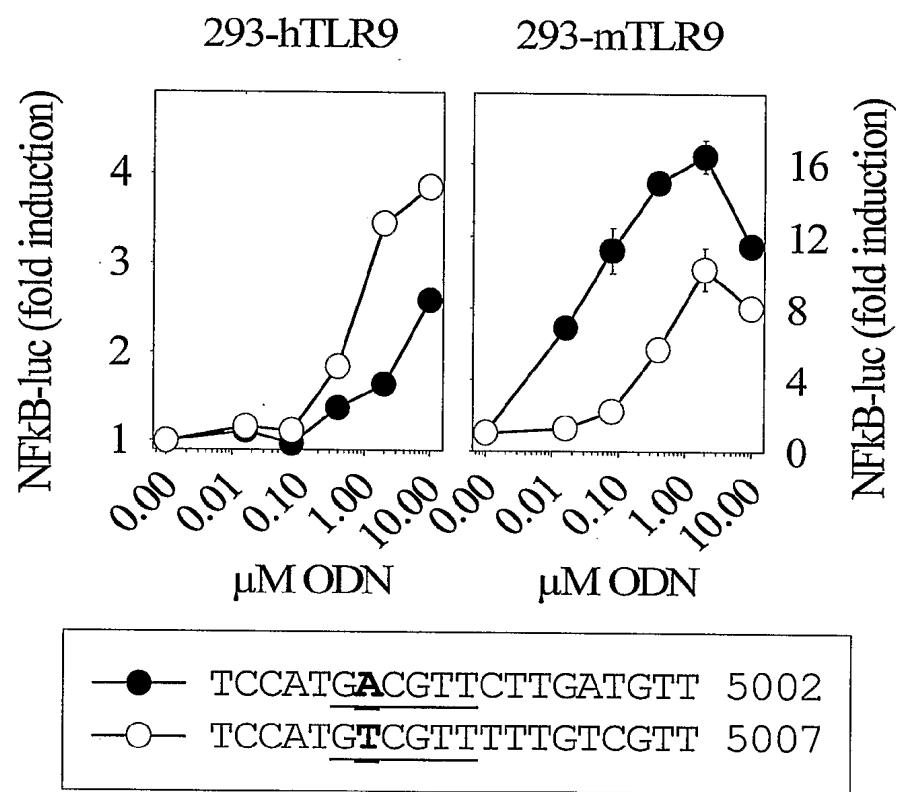


Figure 14

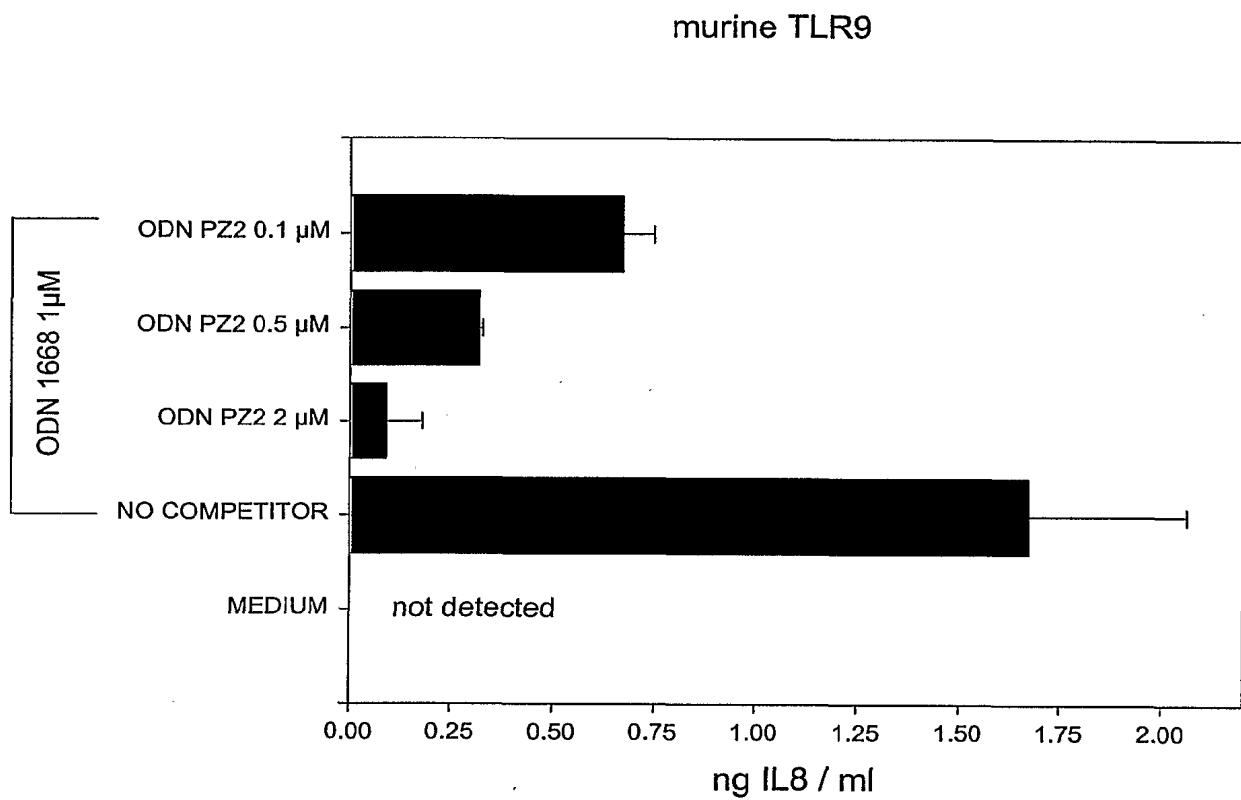


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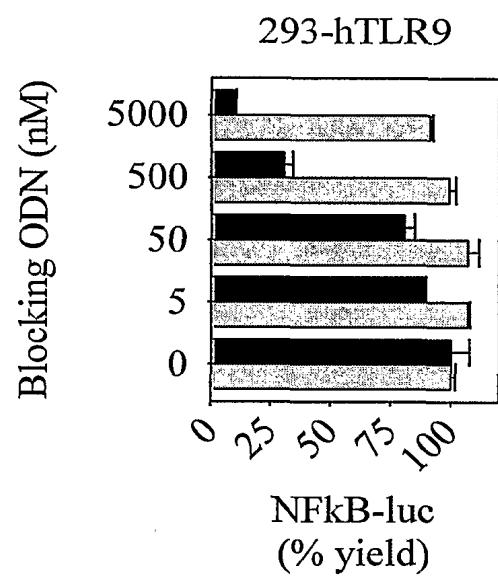


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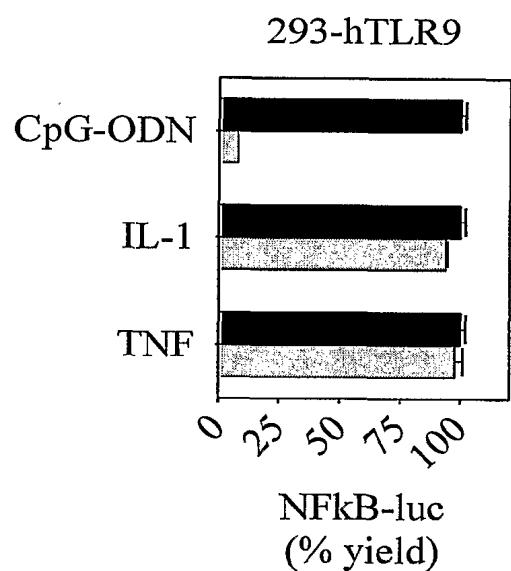


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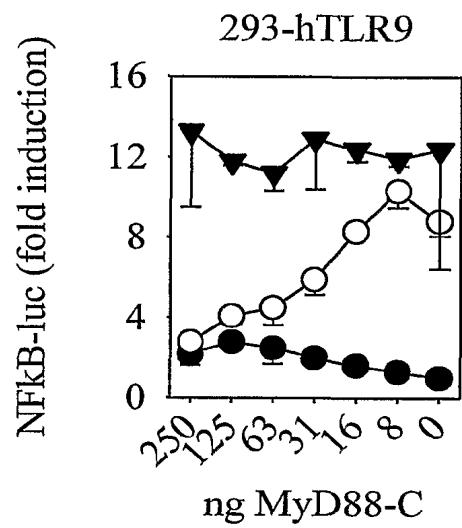


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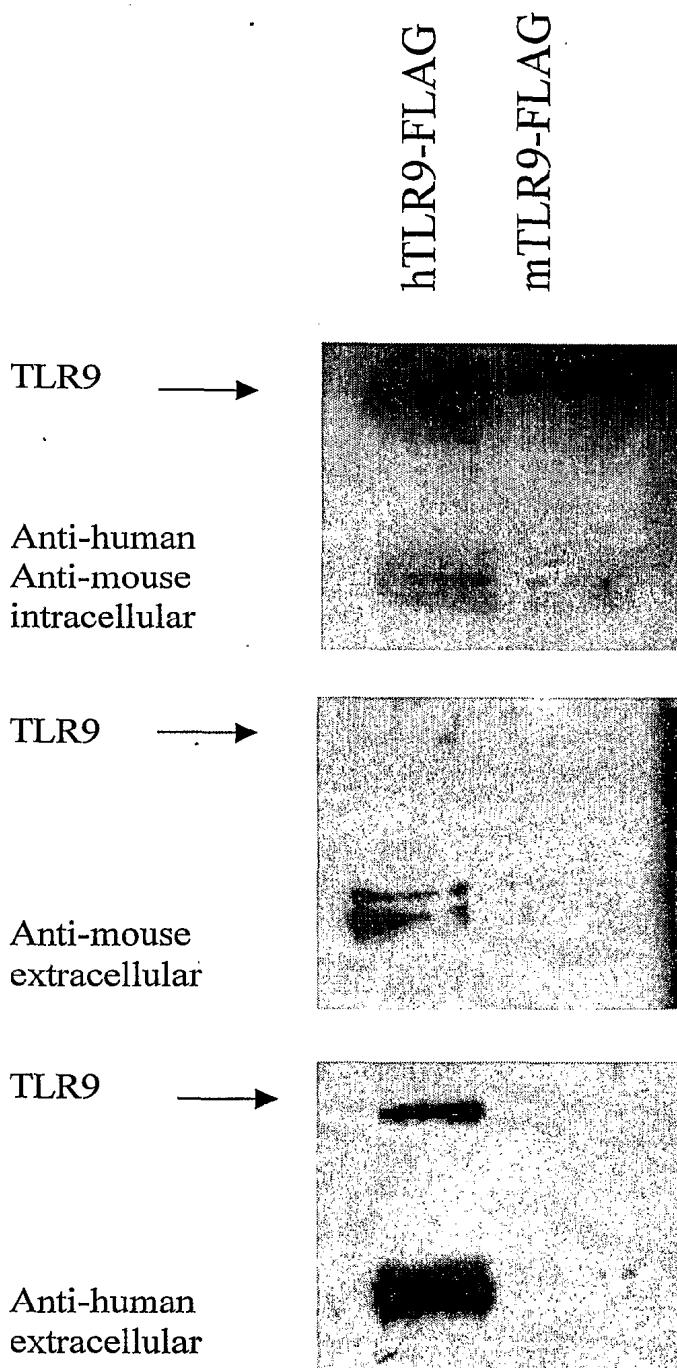


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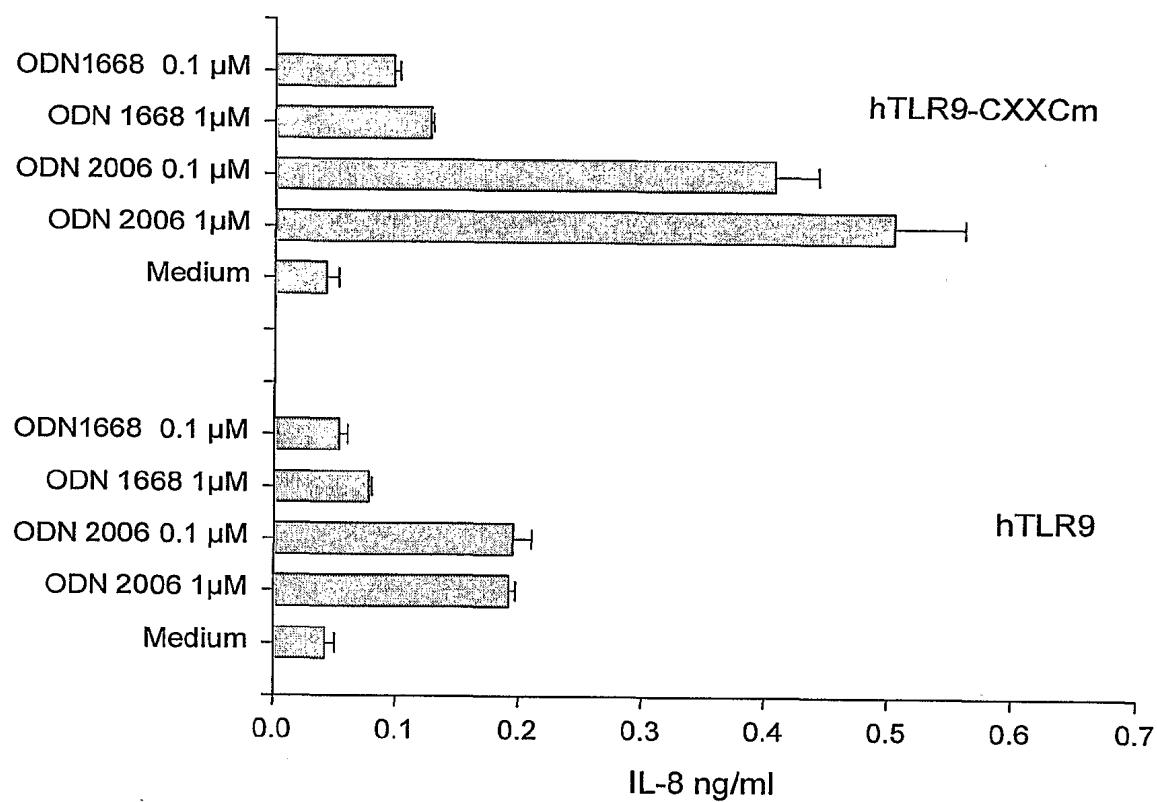


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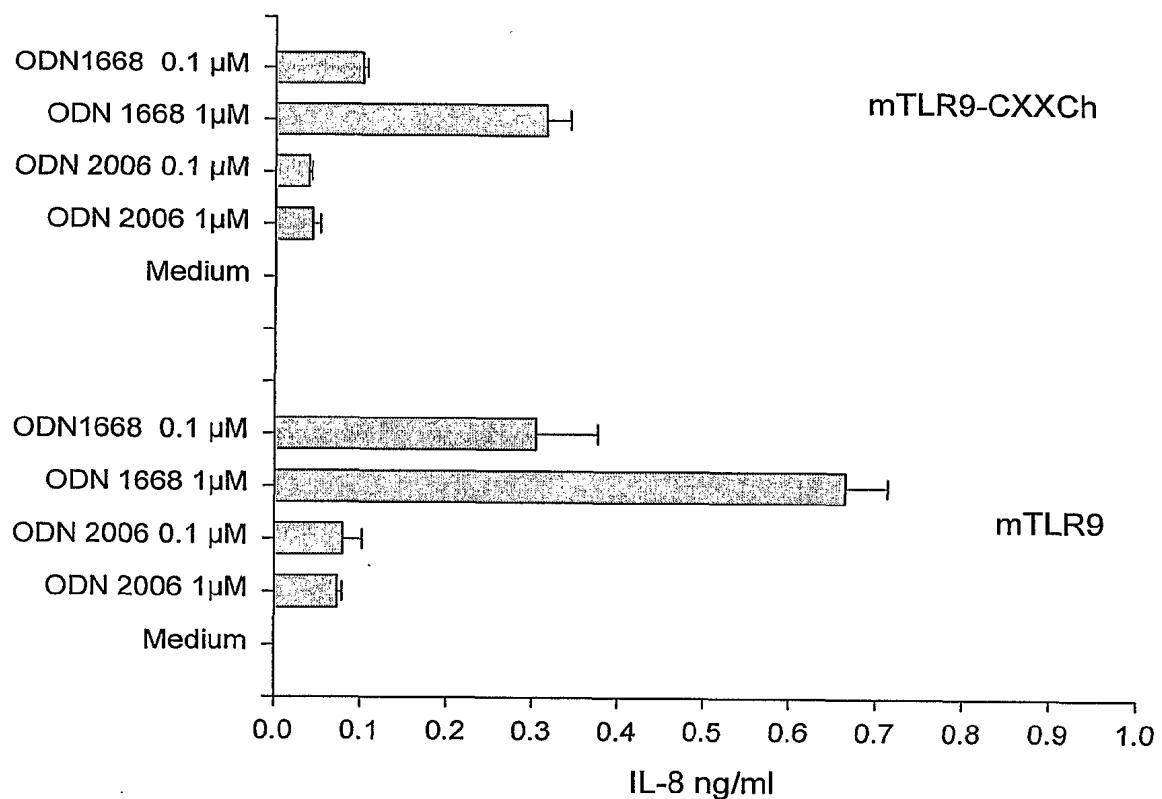


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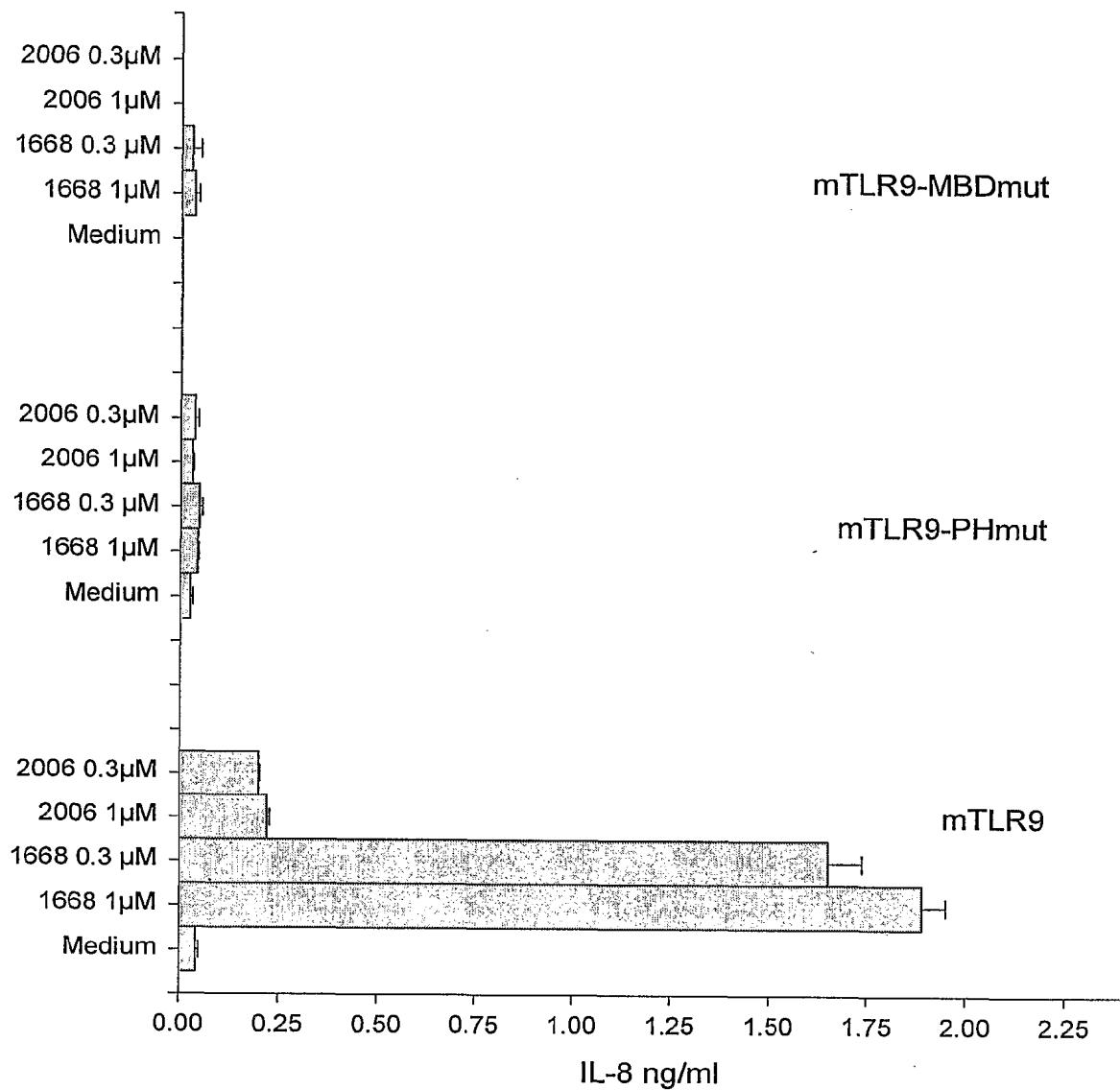


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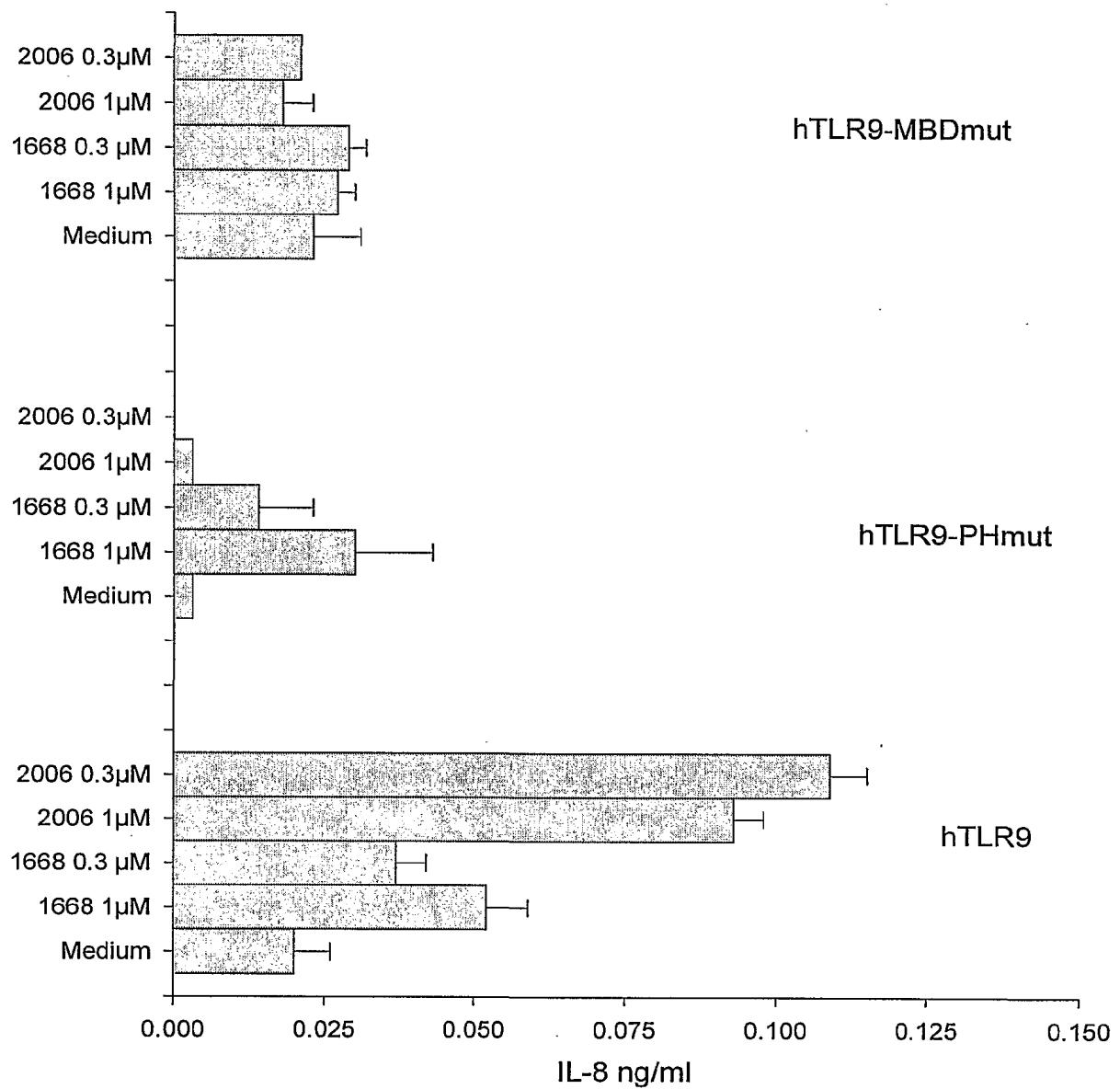


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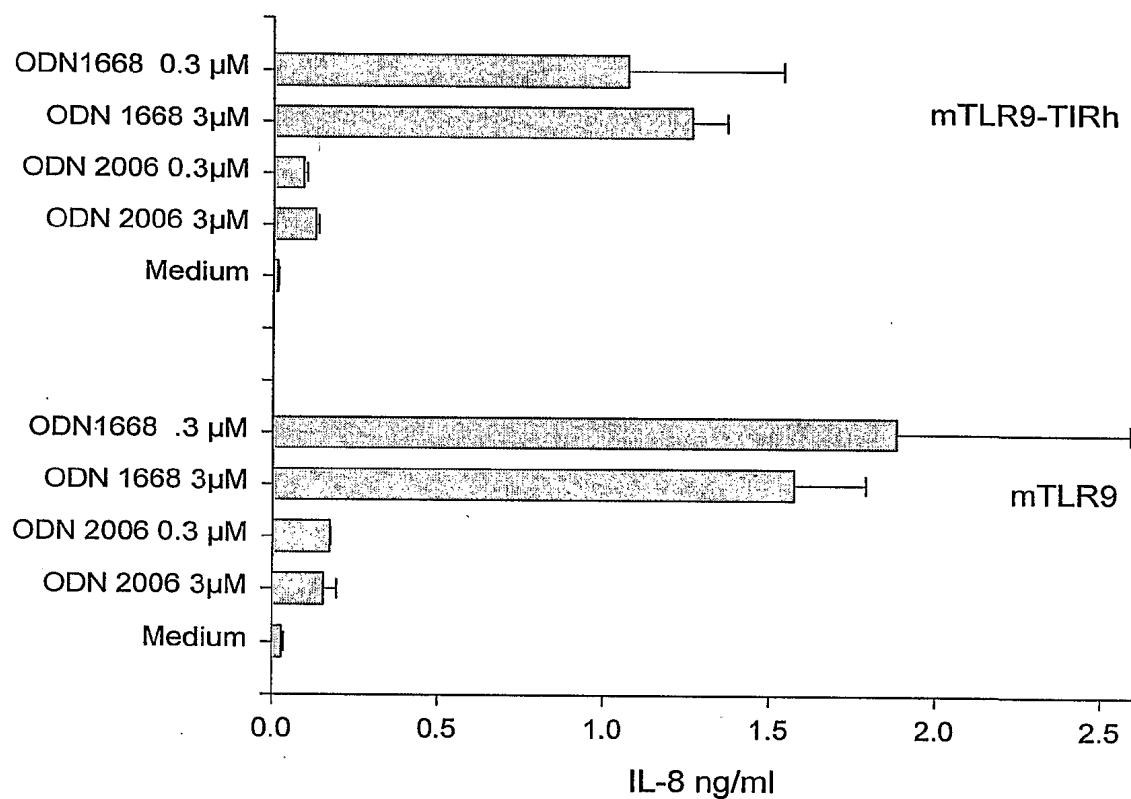


Figure 24

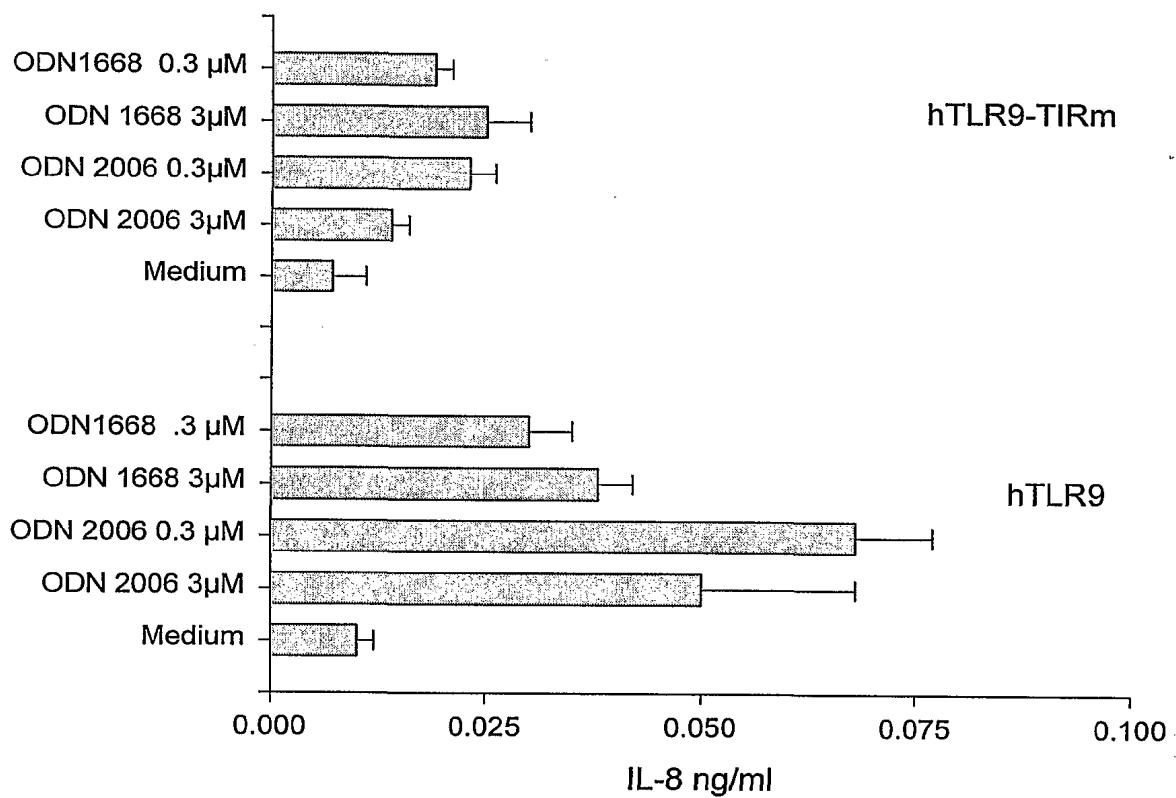


Figure 25

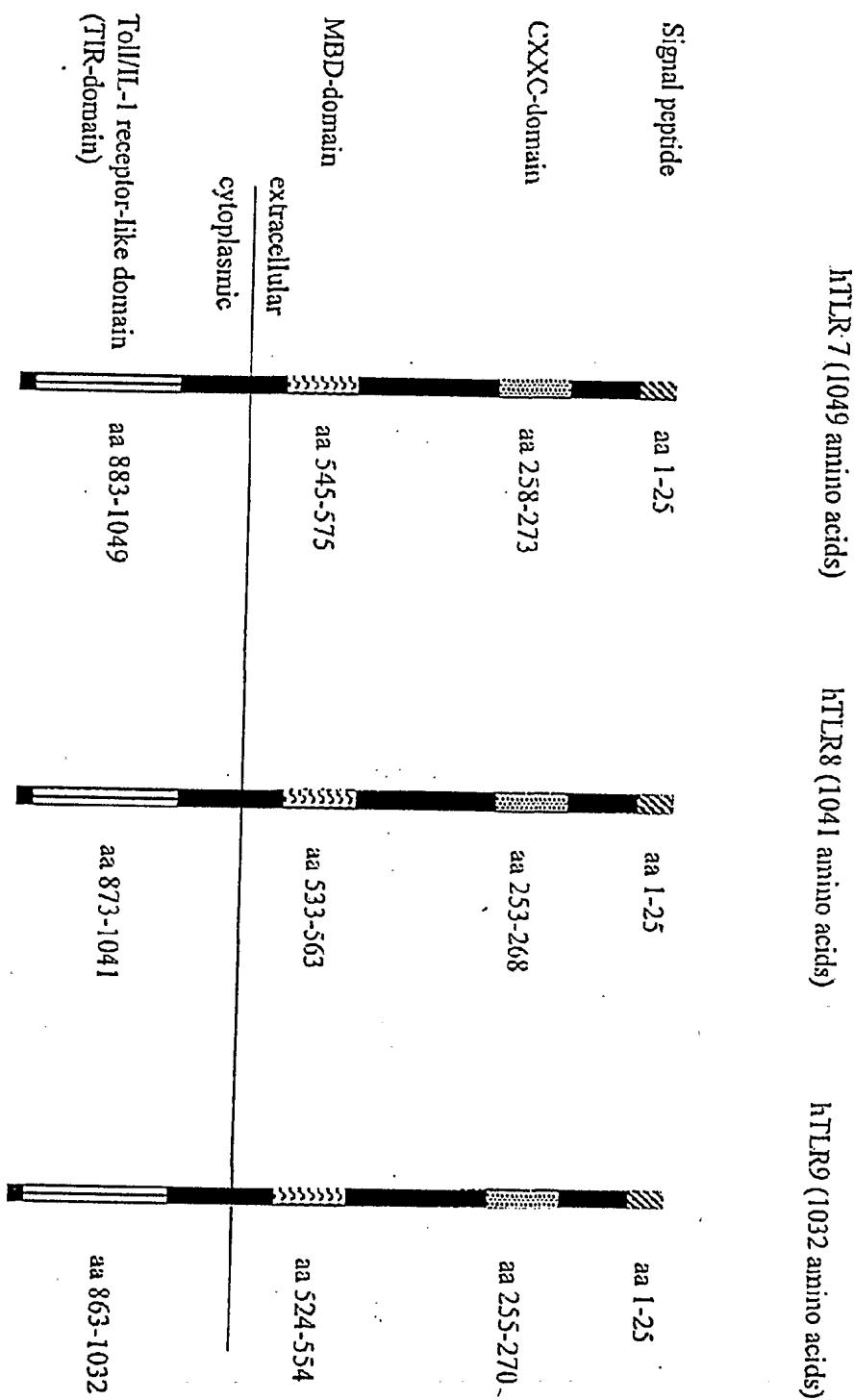


Figure 26

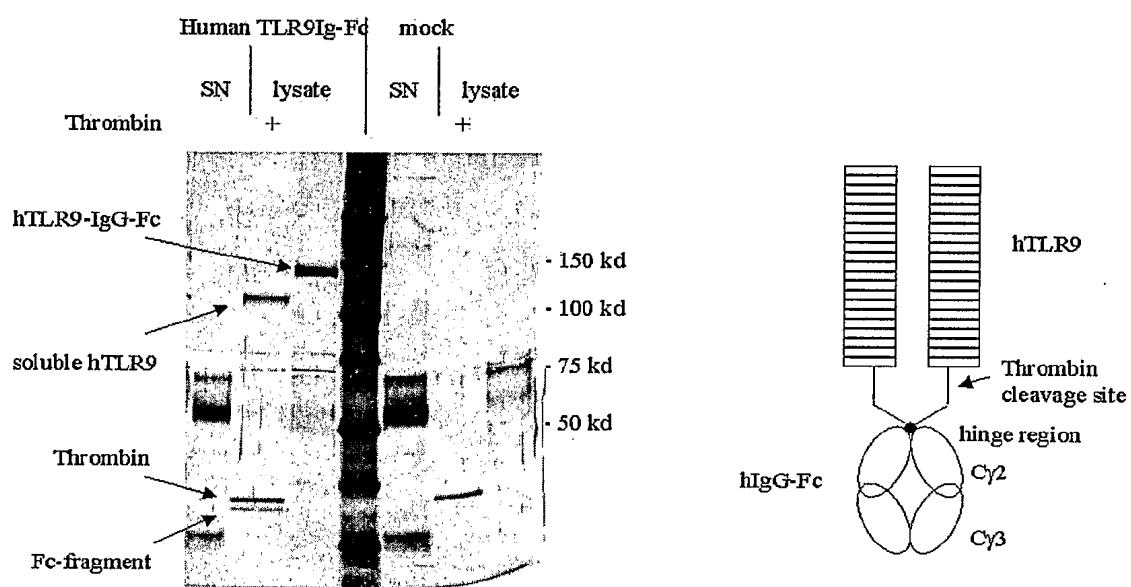


Figure 27

## SEQUENCE LISTING

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<211> 373	
<212> DNA	
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<210> 8	
<211> 489	
<212> DNA	
<213> unknown	
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<210> 9	
<211> 462	
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gccgcagaga	tggtcagta	taggcaccac	catgcccacg	gccacagcca	agagtgaaag	240
gccaaaggcag	tcccaagaga	ggacctcata	caggcacagc	cgcaggctct	gcgcgaagat	300
gctacggccc	tgtagctggc	cggggctgcc	acacttcaca	ccattagcca	ggccaggcac	360
cttggctctgc	acctccaaca	gtaatgtctac	gaaggctgcc	ccacaggcac	agtgcagagg	420
gttgccttctc	acgtctagaa	ctgtcaggtt	catcacaatg	gg		462

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<211> 1032  
<212> PRT  
<213> unknown

<400> 10					
Met	Val	Leu	Arg	Arg	Arg
1			5		10
Ala	Ala	Val	Leu	Ala	Glu
20			25		30
Leu	Pro	Cys	Glu	Leu	Lys
35			40		45
Phe	Leu	Lys	Ser	Val	Pro
50			Arg		Phe
Ile	Thr	Arg	Leu	Ser	Leu
65			70		75
Ser	Asp	Phe	Val	His	Leu
85			Asn		Asn
Asn	Cys	Pro	Pro	Thr	Gly
100					Leu
Thr	Ile	Glu	Pro	Arg	Thr
115					Phe
Asn	Leu	Ser	Tyr	Asn	Gly
130			Ile		Ile
Leu	Val	Asn	Leu	Ser	Thr
145			Leu		Val
Asn	Ser	Leu	Ala	Gly	Leu
165			Tyr		Ser
Asn	Cys	Tyr	Tyr	Lys	Asn
180			Asn		Pro
Gly	Ala	Leu	Leu	Gly	Cys
195			Leu		Thr
Asn	Asn	Leu	Thr	Lys	Val
210			Pro		Pro
Leu	Leu	Val	Arg	Gln	Leu
225			Val		Pro
Ala	Asn	Leu	Thr	Leu	Ser
245			Arg		Asn
Arg	Cys	Asp	His	Ala	Pro
260			Asn		Asn
Leu	His	Leu	His	Pro	Pro
275			Glu		Cys
Leu	Val	Leu	Lys	Thr	Gly
290			Asp		Gly
Gln	Gly	Leu	Val	Ser	Asn
305			Asn		Phe
Tyr	Glu	Ser	Ile	Leu	Asn
325			Asn		Leu
Arg	Lys	Leu	Asn	Leu	Asn
340			Ser		Phe
Arg	Leu	His	Leu	Asn	Arg
355			Ser		Leu
Leu	Asn	Met	Asn	Gly	Gly
				Ile	Ile
				Phe	Phe
				Arg	Arg
				Ser	Ser
				Leu	Leu
				Asn	Asn
				Lys	Lys
				Tyr	Tyr
				Thr	Thr
				Leu	Leu

370	375	380
Arg Trp Leu Ala Asp	Leu Pro Lys Leu His	Thr Leu His Leu Gln Met
385	390	395 400
Asn Phe Ile Asn Gln	Ala Gln Leu Ser	Ile Phe Gly Thr Phe Arg Ala
	405	410 415
Leu Arg Phe Val Asp	Leu Ser Asp Asn Arg	Ile Ser Gly Pro Ser Thr
	420	425 430
Leu Ser Glu Ala Thr	Pro Glu Glu Ala Asp	Asp Ala Glu Gln Glu Glu
	435	440 445
Leu Leu Ser Ala Asp	Pro His Pro Ala Pro	Leu Ser Thr Pro Ala Ser
	450	455 460
Lys Asn Phe Met Asp	Arg Cys Lys Asn Phe	Lys Phe Thr Met Asp Leu
	465	470 475 480
Ser Arg Asn Asn	Leu Val Thr Ile Lys	Pro Glu Met Phe Val Asn Leu
	485	490 495
Ser Arg Leu Gln Cys	Leu Ser Leu Ser His Asn Ser	Ile Ala Gln Ala
	500	505 510
Val Asn Gly Ser Gln	Phe Leu Pro Leu Thr Asn	Leu Gln Val Leu Asp
	515	520 525
Leu Ser His Asn Lys	Leu Asp Leu Tyr His Trp	Lys Ser Phe Ser Glu
	530	535 540
Leu Pro Gln Leu Gln	Ala Leu Asp Leu Ser	Tyr Asn Ser Gln Pro Phe
	545	550 555 560
Ser Met Lys Gly	Ile Gly His Asn Phe	Ser Phe Val Ala His Leu Ser
	565	570 575
Met Leu His Ser	Leu Ser Leu Ala His Asn Asp	Ile His Thr Arg Val
	580	585 590
Ser Ser His Leu Asn	Ser Asn Ser Val Arg	Phe Leu Asp Phe Ser Gly
	595	600 605
Asn Gly Met Gly Arg	Met Trp Asp Glu Gly	Gly Leu Tyr Leu His Phe
	610	615 620
Phe Gln Gly Leu Ser	Gly Leu Leu Lys	Leu Asp Leu Ser Gln Asn Asn
	625	630 635 640
Leu His Ile Leu Arg	Pro Gln Asn Leu Asp	Asn Leu Pro Lys Ser Leu
	645	650 655
Lys Leu Leu Ser	Leu Arg Asp Asn Tyr	Leu Ser Phe Phe Asn Trp Thr
	660	665 670
Ser Leu Ser Phe Leu	Pro Asn Leu Glu Val	Leu Asp Leu Ala Gly Asn
	675	680 685
Gln Leu Lys Ala	Leu Thr Asn Gly	Thr Leu Pro Asn Gly Thr Leu Leu
	690	695 700
Gln Lys Leu Asp Val	Ser Ser Asn Ser	Ile Val Ser Val Val Pro Ala
	705	710 715 720
Phe Phe Ala Leu	Ala Val Glu Leu Lys	Glu Val Asn Leu Ser His Asn
	725	730 735
Ile Leu Lys Thr	Val Asp Arg Ser	Trp Phe Gly Pro Ile Val Met Asn
	740	745 750
Leu Thr Val Leu Asp	Val Arg Ser Asn	Pro Leu His Cys Ala Cys Gly
	755	760 765
Ala Ala Phe Val Asp	Leu Leu Glu Val	Gln Thr Lys Val Pro Gly
	770	775 780
Leu Ala Asn Gly Val	Lys Cys Gly Ser	Pro Gly Gln Leu Gln Gly Arg
	785	790 795 800
Ser Ile Phe Ala Gln	Asp Leu Arg Leu Cys	Leu Asp Glu Val Leu Ser
	805	810 815
Trp Asp Cys Phe Gly	Leu Ser Leu Leu Ala Val	Ala Val Gly Met Val
	820	825 830
Val Pro Ile Leu His	His Leu Cys Gly	Trp Asp Val Trp Tyr Cys Phe
	835	840 845

His Leu Cys Leu Ala Trp Leu Pro Leu Leu Ala Arg Ser Arg Arg Ser  
 850 855 860  
 Ala Gln Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Ala Gln  
 865 870 875 880  
 Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Val Arg Leu Glu  
 885 890 895  
 Glu Arg Arg Gly Arg Arg Ala Leu Arg Leu Cys Leu Glu Asp Arg Asp  
 900 905 910  
 Trp Leu Pro Gly Gln Thr Leu Phe Glu Asn Leu Trp Ala Ser Ile Tyr  
 915 920 925  
 Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser  
 930 935 940  
 Gly Leu Leu Arg Thr Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu  
 945 950 955 960  
 Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Arg Pro Asp Ala His  
 965 970 975  
 Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val  
 980 985 990  
 Leu Phe Trp Pro Gln Gln Pro Asn Gly Gln Gly Phe Trp Ala Gln  
 995 1000 1005  
 Leu Ser Thr Ala Leu Thr Arg Asp Asn Arg His Phe Tyr Asn Gln Asn  
 1010 1015 1020  
 Phe Cys Arg Gly Pro Thr Ala Glu  
 1025 1030

<210> 11  
 <211> 1032  
 <212> PRT  
 <213> unknown

<400> 11  
 Met Gly Phe Cys Arg Ser Ala Leu His Pro Leu Ser Leu Leu Val Gln  
 1 5 10 15  
 Ala Ile Met Leu Ala Met Thr Leu Ala Leu Gly Thr Leu Pro Ala Phe  
 20 25 30  
 Leu Pro Cys Glu Leu Gln Pro His Gly Leu Val Asn Cys Asn Trp Leu  
 35 40 45  
 Phe Leu Lys Ser Val Pro His Phe Ser Met Ala Ala Pro Arg Gly Asn  
 50 55 60  
 Val Thr Ser Leu Ser Leu Ser Asn Arg Ile His His Leu His Asp  
 65 70 75 80  
 Ser Asp Phe Ala His Leu Pro Ser Leu Arg His Leu Asn Leu Lys Trp  
 85 90 95  
 Asn Cys Pro Pro Val Gly Leu Ser Pro Met His Phe Pro Cys His Met  
 100 105 110  
 Thr Ile Glu Pro Ser Thr Phe Leu Ala Val Pro Thr Leu Glu Glu Leu  
 115 120 125  
 Asn Leu Ser Tyr Asn Asn Ile Met Thr Val Pro Ala Leu Pro Lys Ser  
 130 135 140  
 Leu Ile Ser Leu Ser Leu Ser His Thr Asn Ile Leu Met Leu Asp Ser  
 145 150 155 160  
 Ala Ser Leu Ala Gly Leu His Ala Leu Arg Phe Leu Phe Met Asp Gly  
 165 170 175  
 Asn Cys Tyr Tyr Lys Asn Pro Cys Arg Gln Ala Leu Glu Val Ala Pro  
 180 185 190  
 Gly Ala Leu Leu Gly Leu Gly Asn Leu Thr His Leu Ser Leu Lys Tyr  
 195 200 205  
 Asn Asn Leu Thr Val Val Pro Arg Asn Leu Pro Ser Ser Leu Glu Tyr  
 210 215 220

Leu Leu Leu Ser Tyr Asn Arg Ile Val Lys Leu Ala Pro Glu Asp Leu  
 225 230 235 240  
 Ala Asn Leu Thr Ala Leu Arg Val Leu Asp Val Gly Gly Asn Cys Arg  
 245 250 255  
 Arg Cys Asp His Ala Pro Asn Pro Cys Met Glu Cys Pro Arg His Phe  
 260 265 270  
 Pro Gln Leu His Pro Asp Thr Phe Ser His Leu Ser Arg Leu Glu Gly  
 275 280 285  
 Leu Val Leu Lys Asp Ser Ser Leu Ser Trp Leu Asn Ala Ser Trp Phe  
 290 295 300  
 Arg Gly Leu Gly Asn Leu Arg Val Leu Asp Leu Ser Glu Asn Phe Leu  
 305 310 315 320  
 Tyr Lys Cys Ile Thr Lys Thr Lys Ala Phe Gln Gly Leu Thr Gln Leu  
 325 330 335  
 Arg Lys Leu Asn Leu Ser Phe Asn Tyr Gln Lys Arg Val Ser Phe Ala  
 340 345 350  
 His Leu Ser Leu Ala Pro Ser Phe Gly Ser Leu Val Ala Leu Lys Glu  
 355 360 365  
 Leu Asp Met His Gly Ile Phe Phe Arg Ser Leu Asp Glu Thr Thr Leu  
 370 375 380  
 Arg Pro Leu Ala Arg Leu Pro Met Leu Gln Thr Leu Arg Leu Gln Met  
 385 390 395 400  
 Asn Phe Ile Asn Gln Ala Gln Leu Gly Ile Phe Arg Ala Phe Pro Gly  
 405 410 415  
 Leu Arg Tyr Val Asp Leu Ser Asp Asn Arg Ile Ser Gly Ala Ser Glu  
 420 425 430  
 Leu Thr Ala Thr Met Gly Glu Ala Asp Gly Gly Glu Lys Val Trp Leu  
 435 440 445  
 Gln Pro Gly Asp Leu Ala Pro Ala Pro Val Asp Thr Pro Ser Ser Glu  
 450 455 460  
 Asp Phe Arg Pro Asn Cys Ser Thr Leu Asn Phe Thr Leu Asp Leu Ser  
 465 470 475 480  
 Arg Asn Asn Leu Val Thr Val Gln Pro Glu Met Phe Ala Gln Leu Ser  
 485 490 495  
 His Leu Gln Cys Leu Arg Leu Ser His Asn Cys Ile Ser Gln Ala Val  
 500 505 510  
 Asn Gly Ser Gln Phe Leu Pro Leu Thr Gly Leu Gln Val Leu Asp Leu  
 515 520 525  
 Ser Arg Asn Lys Leu Asp Leu Tyr His Glu His Ser Phe Thr Glu Leu  
 530 535 540  
 Pro Arg Leu Glu Ala Leu Asp Leu Ser Tyr Asn Ser Gln Pro Phe Gly  
 545 550 555 560  
 Met Gln Gly Val Gly His Asn Phe Ser Phe Val Ala His Leu Arg Thr  
 565 570 575  
 Leu Arg His Leu Ser Leu Ala His Asn Asn Ile His Ser Gln Val Ser  
 580 585 590  
 Gln Gln Leu Cys Ser Thr Ser Leu Arg Ala Leu Asp Phe Ser Gly Asn  
 595 600 605  
 Ala Leu Gly His Met Trp Ala Glu Gly Asp Leu Tyr Leu His Phe Phe  
 610 615 620  
 Gln Gly Leu Ser Gly Leu Ile Trp Leu Asp Leu Ser Gln Asn Arg Leu  
 625 630 635 640  
 His Thr Leu Leu Pro Gln Thr Leu Arg Asn Leu Pro Lys Ser Leu Gln  
 645 650 655  
 Val Leu Arg Leu Arg Asp Asn Tyr Leu Ala Phe Phe Lys Trp Trp Ser  
 660 665 670  
 Leu His Phe Leu Pro Lys Leu Glu Val Leu Asp Leu Ala Gly Asn Arg  
 675 680 685  
 Leu Lys Ala Leu Thr Asn Gly Ser Leu Pro Ala Gly Thr Arg Leu Arg

690	695	700
Arg Leu Asp Val Ser Cys Asn Ser Ile Ser Phe Val Ala Pro Gly Phe		
705	710	715
Phe Ser Lys Ala Lys Glu Leu Arg Glu Leu Asn Leu Ser Ala Asn Ala		720
725	730	735
Leu Lys Thr Val Asp His Ser Trp Phe Gly Pro Leu Ala Ser Ala Leu		
740	745	750
Gln Ile Leu Asp Val Ser Ala Asn Pro Leu His Cys Ala Cys Gly Ala		
755	760	765
Ala Phe Met Asp Phe Leu Leu Glu Val Gln Ala Ala Val Pro Gly Leu		
770	775	780
Pro Ser Arg Val Lys Cys Gly Ser Pro Gly Gln Leu Gln Gly Leu Ser		
785	790	795
Ile Phe Ala Gln Asp Leu Arg Leu Cys Leu Asp Glu Ala Leu Ser Trp		800
805	810	815
Asp Cys Phe Ala Leu Ser Leu Leu Ala Val Ala Leu Gly Leu Gly Val		
820	825	830
Pro Met Leu His His Leu Cys Gly Trp Asp Leu Trp Tyr Cys Phe His		
835	840	845
Leu Cys Leu Ala Trp Leu Pro Trp Arg Gly Arg Gln Ser Gly Arg Asp		
850	855	860
Glu Asp Ala Leu Pro Tyr Asp Ala Phe Val Val Phe Asp Lys Thr Gln		
865	870	875
Ser Ala Val Ala Asp Trp Val Tyr Asn Glu Leu Arg Gly Gln Leu Glu		880
885	890	895
Glu Cys Arg Gly Arg Trp Ala Leu Arg Leu Cys Leu Glu Glu Arg Asp		
900	905	910
Trp Leu Pro Gly Lys Thr Leu Phe Glu Asn Leu Trp Ala Ser Val Tyr		
915	920	925
Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr Asp Arg Val Ser		
930	935	940
Gly Leu Leu Arg Ala Ser Phe Leu Leu Ala Gln Gln Arg Leu Leu Glu		
945	950	955
Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Ser Pro Asp Gly Arg		960
965	970	975
Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys Arg Gln Ser Val		
980	985	990
Leu Leu Trp Pro His Gln Pro Ser Gly Gln Arg Ser Phe Trp Ala Gln		
995	1000	1005
Leu Gly Met Ala Leu Thr Arg Asp Asn His His Phe Tyr Asn Arg Asn		
1010	1015	1020
Phe Cys Gln Gly Pro Thr Ala Glu		
1025	1030	

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 <211> 178  
 <212> PRT  
 <213> unknown

<400> 12		
Leu Asn Leu Ser Phe Asn Tyr Arg Lys Lys Val Ser Phe Ala Arg Leu		
1	5	10
His Leu Ala Ser Ser Phe Lys Asn Leu Val Ser Leu Gln Glu Leu Asn		15
20	25	30
Met Asn Gly Ile Phe Phe Arg Leu Leu Asn Lys Tyr Thr Leu Arg Trp		
35	40	45
Leu Ala Asp Leu Pro Lys Leu His Thr Leu His Leu Gln Met Asn Phe		
50	55	60
Ile Asn Gln Ala Gln Leu Ser Ile Phe Gly Thr Phe Arg Ala Leu Arg		

65	70	75	80
Phe Val Asp Leu Ser Asp Asn Arg Ile Ser Gly Pro Ser Thr Leu Ser			
85	90	95	
Glu Ala Thr Pro Glu Glu Ala Asp Asp Ala Glu Gln Glu Glu Leu Leu			
100	105	110	
Ser Ala Asp Pro His Pro Ala Pro Leu Ser Thr Pro Ala Ser Lys Asn			
115	120	125	
Phe Met Asp Arg Cys Lys Asn Phe Lys Phe Asn Met Asp Leu Ser Arg			
130	135	140	
Asn Asn Leu Val Thr Ile Thr Ala Glu Met Phe Val Asn Leu Ser Arg			
145	150	155	160
Leu Gln Cys Leu Ser Leu Ser His Asn Ser Ile Ala Gln Ala Val Asn			
165	170	175	
Gly Ser			

<210> 13  
<211> 95  
<212> PRT  
<213> unknown

<400> 13			
Ala His Thr Asp Arg Val Ser Gly Leu Leu Arg Thr Ser Phe Leu Leu			
1	5	10	15
Ala Gln Gln Arg Leu Leu Glu Asp Arg Lys Asp Val Val Val Leu Val			
20	25	30	
Ile Leu Arg Pro Asp Ala Xaa Pro Ser Arg Tyr Val Arg Leu Arg Gln			
35	40	45	
Arg Leu Cys Arg Gln Ser Val Leu Phe Trp Pro Gln Arg Pro Asn Gly			
50	55	60	
Gln Gly Gly Phe Trp Ala Gln Leu Ser Thr Ala Leu Thr Arg Asp Asn			
65	70	75	80
Arg His Phe Tyr Asn Gln Asn Phe Cys Arg Gly Pro Thr Ala Glu			
85	90	95	

<210> 14  
<211> 123  
<212> PRT  
<213> unknown

<400> 14			
Glu Asp Arg Asp Trp Leu Pro Gly Gln Thr Leu Phe Glu Asn Leu Trp			
1	5	10	15
Ala Ser Ile Tyr Gly Ser Arg Lys Thr Leu Phe Val Leu Ala His Thr			
20	25	30	
Asp Arg Val Ser Gly Leu Leu Arg Thr Ser Phe Leu Leu Ala Gln Gln			
35	40	45	
Arg Leu Leu Glu Asp Arg Lys Asp Val Val Val Leu Val Ile Leu Arg			
50	55	60	
Pro Asp Ala His Arg Ser Arg Tyr Val Arg Leu Arg Gln Arg Leu Cys			
65	70	75	80
Arg Gln Ser Val Leu Phe Trp Pro Gln Gln Pro Asn Gly Gln Gly			
85	90	95	
Phe Trp Ala Gln Leu Ser Thr Ala Leu Thr Arg Asp Asn Arg His Phe			
100	105	110	
Tyr Asn Gln Asn Phe Cys Arg Gly Pro Thr Ala			
115	120		

<210> 15

<211> 162  
<212> PRT  
<213> unknown

<400> 15

Tyr Asn Ser Gln Pro Phe Ser Met Lys Gly Ile Gly His Asn Phe Ser  
1 5 10 15  
Phe Val Thr His Leu Ser Met Leu Gln Ser Leu Ser Leu Ala His Asn  
20 25 30  
Asp Ile His Thr Arg Val Ser Ser His Leu Asn Ser Asn Ser Val Arg  
35 40 45  
Phe Leu Asp Phe Ser Gly Asn Gly Met Gly Arg Met Trp Asp Glu Gly  
50 55 60  
Gly Leu Tyr Leu His Phe Phe Gln Gly Leu Ser Gly Val Leu Lys Leu  
65 70 75 80  
Asp Leu Ser Gln Asn Asn Leu His Ile Leu Arg Pro Gln Asn Leu Asp  
85 90 95  
Asn Leu Pro Lys Ser Leu Lys Leu Leu Ser Leu Arg Asp Asn Tyr Leu  
100 105 110  
Ser Phe Phe Asn Trp Thr Ser Leu Ser Phe Leu Pro Asn Leu Glu Val  
115 120 125  
Leu Asp Leu Ala Gly Asn Gln Leu Lys Ala Leu Thr Asn' Gly Thr Leu  
130 135 140  
Pro Asn Gly Thr Leu Leu Gln Lys Leu Asp Val Ser Ser Asn Ser Ile  
145 150 155 160  
Val Ser

<210> 16

<400> 16

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